Immuno-hematopoietic cytokines in teleost fish

DOCTORAL DISSERTATION

IMMUNO-HEMATOPOIETIC CYTOKINES IN TELEOST FISH

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Graduate School of Marine Science and Technology
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(for hard cover)

IMMUNO-HEMATOPOIETIC CYTOKINES IN TELEOST FISH

（魚類白血球の増殖・分化に関与するサイトカイン）

A Dissertation presented

for the

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Applied Marine Biosciences

by

MUDJEKEEWIS DALISAY SANTOS

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Laboratory of Genome Science
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DECLARATION

I hereby declare that this dissertation has been composed by myself and is a result of my own investigation. It has never been accepted, nor submitted for any other degrees. All sources of information have been duly acknowledged.

Mudjekeewis D. Santos

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And to God Almighty, in whom all things become possible!

ABBREVIATIONS

BAC	- Bacterial Artificial Chromosome
BLAST	- Basic Local Alignment and Search Tool
CBD	- Cytokine Binding Domain
cDNA	- complementary DNA
CFU	- Colony Forming Unit
CNE	- Conserved noncoding elements
CNTF	- Ciliary neutrophic factor
CNTFR	- Ciliary neutrophic factor receptor
CT	- Cardiotropin
ConA	- Concanavalin A
CSF1	- Macrophage colony-stimulating factor
CSF1R	- Macrophage colony-stimulating factor receptor
CSF2	- Granulocyte-macrophage colony-stimulating factor
CSF2R	- Granulocyte-macrophage colony-stimulating factor receptor
CSF3	- Granulocyte colony-stimulating factor
CSF3R	- Granulocyte colony-stimulating factor receptor
DDBJ	- DNA Databank of Japan
EST	- Expressed sequence tag
FBS	- Fetal bovine serum
FN3	- Fibronectin Type III
GP130	- Glycoprotein 130
HINAE	- Hirame Natural Embryo cell line
HIRRV	- Hirame rhabdovirus
IFN	- Interferon
IL	- Interleukin
IL-6	- Interleukin 6
IL-6Rα	- Interleukin 6 receptor alpha
IL-11	- Interleukin 11
IL-11Rα	- Interleukin 11 receptor alpha
Jak	- Janus kinase
JfGPH	- Japanese flounder GP130 Homologue
kDa	- kiloDaltons
LIF	- Leukemia inhibitory factor
LIFR	- Leukemia inhibitory factor receptor
LPS	- Lipopolysaccharide
Mab	- Monoclonal antibody
MGF	- Myelomonocytic Growth Factor
MSH	- M17 Homologue
MP	- Maximum Parsimony
Mx	- Myxovirus resistance-1 protein
Myr	- Million years ago
NCC	- Non-specific cytotoxic cells
NITR	- Novel immune type receptor

NJ	- Neighbor-joining
NP	- Neuropoietin
ORF	- Open reading frame
OSM	- Oncostatin M
OSMR	- Oncostatin M receptor
Pab	- Polyclonal antibody
PAMP	- Pathogen-associated molecular patterns
PBS	- Phosphate buffer saline
PBL	- Peripheral blood leukocytes
PCR	- Polymerase Chain Reaction
PDB	- Protein Data Bank
PG	- Peptidoglycan
PMA	- Phorbol Myristate Acetate
Poly I:C	- Polyinosinic:polycytidylic acid
PRR	- Pathogen recognition receptor
RT-PCR	- Reverse transcriptase PCR
SCOP	- Structural Classification of Proteins
STAT	- Signal transducer and activation of transcription
SOCS	- Supressor of cytokine signaling
TCID	- Tissue Culture Infectious Dose
TNF	- Tumor Necrosis Factor
Tyk	- Tyrosine kinase
UPGMA	- Unweighted Pair Group Method with Arithmetic mean
UTR	- Untranslated region
WGD	- Whole Genome Duplication

GENERAL ABSTRACT

Teleost fish exhibits tremendous diversity at the species, cellular and molecular levels. This diversity is reflected in its immune and hematopoietic (blood) systems, where both a general conservation as well as divergence of molecules and processes is observable between these group of species and higher vertebrates. In particular, the genetic programs underlying the molecular mechanisms of immuno-hematopoiesis are said to be well conserved, although, recent studies have revealed a number of novel genes that are involved, and with increasing evidence of whole genome duplication (WGD), it is becoming clear that the molecular mechanisms in teleost fish are far more complex than previously thought, which have far reaching implications in teleost fish immuno-hematopoietic studies. Among the immuno-hematopoietic genes that are relatively unknown are the cytokines and their cognate receptors that have been shown in mammals to play a critical role in hematopoiesis, particularly in the development and maturation of lymphocytes and leukocytes. Foremost is the long chain, 4 α-helical, IL6-cytokine subfamily whose members utilize a common glycoprotein 130 (gp130) receptor. Here, we identified, characterized and studied the function of teleost fish IL6-cytokines and cytokine receptor(s) that are putatively involved in immuno-hematopoiesis as well as investigate whether these cytokines, following the WGD, are in duplicates and shows expression and structural divergence. We also developed some tools that could further the study of immuno-hematopoiesis in teleost fish.

Three long chain, α-helical cytokines were fully cloned and characterized from Japanese flounder and identified in fish genomic databases; one is shown to be orthologous to granulocyte colony-stimulating factor (CSF3) (known to be involved in granulopoiesis), another to Interleukin 11 (known to be involved in magakaryocytopoiesis) and another to M17 (an IL6-cytokine present only in fish involved in immune regulation). CSF3 is a glycoprotein cytokine which influences the hematopoiesis of the phagocytic neutrophils and its precursors, and has been used extensively in cancer therapy and for the treatment of neutropenia in mammals. We report the duplicated CSF3 genes from 3 teleost fishes: Japanese flounder, fugu, *Takifugu rubripes* and green spotted pufferfish, *Tetraodon nigroviridis.* Duplicated fugu and pufferfish CSF3-as and CSF3-bs are shown to be the ancestral and duplicate genes, respectively. Moreover, we demonstrate that the Japanese flounder CSF3b gene is at least involved in immunity based on its basal expression in immune-related tissues, and its up-regulation in kidney and peripheral blood leukocytes by LPS, a combination of conA/PMA and by poly I:C, a known interferon inducer. IL-11, on the other hand, is involved in physiological processes including blood production, bone formation and placentation. The IL-11 duplicates (IL11a and IL11b) have been identified in fish with only IL11a from carp and trout have been characterized and analyzed for its expression thus far. Japanese flounder IL11b (*po*IL11b) was confirmed as such by structural and phylogenetic analysis. *po*IL11b doesn't show constitutive expression in tissues of adult fish except for the very slight expression in kidney and spleen, and the very high expression in peripheral blood leukocytes (PBLs). *po*IL11b is transiently up-regulated by

bacterial LPS and increasingly stimulated by the IFN inducer poly I:C in kidney, spleen and peripheral blood leukocytes (PBLs) of adult fish *in vitro*. In addition, it is very slightly stimulated by *Edwardsiella tarda* infection but is highly expressed after hirame rhabdovirus (HIRRV) infection in kidney of juvenile fish, suggesting that *po*IL11b, aside from its role in bacterial infection, is well involved in antiviral responses. Moreover, *po*IL11b structure and expression pattern appears to be slightly distinct and opposite to IL11a, respectively, suggesting a complementation of function of the duplicate fish IL11 genes. The Japanese flounder MSH is closely associated with the IL6 subfamily member M17. MSH had significant identity but contrasting expression with fish M17s. Subsequent *in silico* search and full annotation of the M17 orthologue in zebrafish, *Danio rerio*, MSH orthologues in tiger puffer, green spotted pufferfish and stickleback (*Gastorosteus aculeatus*), as well as structural, synteny comparisons and phylogenetic analysis with known IL6-cytokines, we determined the novelty of the fish MSH. Japanese flounder MSH was observed to be highly expressed in immune-related tissues and are induced by immune stimulants, LPS, poly I:C and PG *in vitro* suggesting its involvement in fish immunity particularly against viral and bacterial agents.

We then studied all existing sequence and expression data for teleost fish IL6-cytokines in relation to the duplication phenomenon. Our analysis confirmed that the 4 teleost IL6-cytokine genes were in duplicates, grouped as Type-A and -B at the subfamily level. The duplicates were found to show contrasting, constitutive expression in tissues between them. Type-As, observed to be more structurally and phylogenetically related to the mammalian orthologs, hence appearing to be the "original" genes, are more involved in antibacterial responses. In contrast, Type-Bs, which showed increased structural divergence, exhibited involvement in both antibacterial and antiviral responses suggesting that the duplicates are undergoing subneofunctionalization. We likewise discovered that the structure of Type-A and -B, while generally conserved, have point differences that correlates to the divergent gene expressions of the IL6-cytokines. Type-Bs, in particular, possessed additional conserved cysteine residues that were strongly predicted to influence the disulfide binding patterns of the protein, and hence its function. These results are the first to be shown in rapidly evolving immune-related genes at the subfamily level in teleost fish and have important implications to the study of its immune system.

We also investigated the cytokine receptors (hematopoietins) that mediate the action of the members of the long chain cytokines, which includes the IL6-cytokines, canonically involved in numerous physiological function. Here we report a novel cytokine receptor termed Japanese flounder glycoprotein 130 homologue or JfGPH, exhibiting the unique type I cytokine receptor motifs i.e. having a cytokine binding domain (CBD) containing two pairs of conserved cysteine (C) residues, a WSXWS motif, 3 fibronectin domains all in the extracellular region. It is also composed of the Jak binding domains Box 1 and Box 2, and a STAT 3 binding motif (Box 3) in the cytoplasmic region suggesting its mediatory role for Janus kinase/Signal transducers and activators of transcription (Jak/STAT) signal pathway. The JfGPH cDNA is about 3 kb encoding 801 amino acid residues with a predicted molecular weight of 90 kDa and its gene has an 11-exon/10-intron architecture. While JfGPH shows significant homology

with the members of type 1 cytokine receptor family including IL6ST (or gp130), IL31α (or GLMR), CSF3R (or GCSFR), LIFR, OSMR, IL12Rβ1 and LEPR, structural and phylogenetic analysis of its protein revealed that it is a novel and an ancestral cytokine receptor found in teleost. JfGPH gene is ubiquitously expressed in Japanese flounder tissues and in a natural embryo (HINAE) cell line showing its critical role in teleost physiological functions similar to gp130 in higher vertebrates. High expression of JfGPH transcripts in immune-related tissues and, in ovary and embryo-derived cell line suggest its role in immune responses, and reproduction/development, respectively. *In vitro* stimulation of spleen, kidney, peripheral blood leukocytes (PBLs) and HINAE revealed that JfGPH is down-regulated by polyinosinic:polycytidylic acid (poly I:C), an interferon (IFN) inducer, suggesting an apparent control of the JfGPH's expression during IFN-induced Jak/STAT signaling.

Lastly, we have successfully developed some tools to further the study of fish immuno-hematopoiesis. We produced a recombinant Japanese flounder CSF3 protein using a fish cell line (HINAE), which is particularly important in producing biologically active recombinant fish proteins for functional analysis and other applications. We likewise produced a useful and cost-effective polyclonal antibody against Japanese flounder IgM, which could be used to evaluate/monitor immunocompetence in fish as well as use these for B cell markers.

Taken together, these results have significantly contributed to the scientific paradigm and study about molecular mechanisms of teleost fish immuno-hematopoiesis.

CHAPTER 1

Genetic and functional diversity of immuno-hematopoiesis in teleost fish: A review

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Genetic and functional diversity of immuno-hematopoiesis in teleost fish: A review

Abstract

Teleost fish exhibits tremendous diversity at the species, cellular and molecular levels. This diversity is reflected in its immune and hematopoietic (blood) systems, where both a general conservation as well as divergence of molecules and processes can be observed between teleost fish and higher vertebrates. In this paper, we review the immuno-hematopoietic system of teleost fish, focusing on molecular mechanisms that are related to white blood cell formation amidst the background of genetic diversity. We conclude the review by mentioning some information gaps and suggesting needed tools for furthering the immuno-hematopoietic studies in teleost fish.

1. Introduction

Teleost fish, the earliest class of vertebrates, comprise the greatest group of vertebrate species whose members can be found even in extreme aquatic habitats. Such remarkable diversity is now believed to be a product of a Whole Genome Duplication (WGD) event that happened early in teleost fish evolution (Volff, 2006).

Perhaps not surprisingly, teleost fish immune and hematopoieitc (blood) systems are also not simple. Earlier, it was believed that these systems, including its genetics, are conserved with higher vertebrates (Plouffe et al., 2005; Thisse and Zon, 2002, Song et al., 2004). However, studies in teleost fish have likewise revealed a number of novel genes that are involved in hematopoiesis and vascularization (Song et al., 2004; Weber et al., 2005). With the WGD, it is becoming clear that the molecular mechanisms in teleost fish are far more complex than previously thought, which have far reaching implications in fish immuno-hematopoietic studies. Among the immuno-hematopoietic genes in teleost fish that are relatively unknown are the hematopoietic cytokines and their cognate hematopoietin receptors. These molecules have been shown in mammals to play a critical

role in hematopoiesis, particularly in the development and maturation of lymphocytes and leukocytes.

In this paper, we review the immune and hematopoietic system of teleost fish. We draw comparisons of these with the known immuno-hematopoitic molecules and mechanisms in mammals, as well as highlight recent and growing molecular functions that are teleost fish-specific.

2. Teleost fish genetic diversity

The WGD that happened early in the life of teleost fish is now increasingly believed to have happened about 350 to 450 million year ago (Myr) and is said to be the main reason for the explosion of the fish diversity at > 23, 500 species (Fig. 1) (Volff 2005). This is possible because it has been postulated that gene duplication created numerous genes in fish with novel or semi-novel functions, otherwise known as "more genes in fish than mammals" concept (Ohno 1970). Supporting this are numerous evidences that now point to a rapid divergence of teleost fish genome. For example, transposable element diversity is much higher in teleost fish than in mammal genomes and therefore mobile sequences apparently undergoes a higher turnover in teleost fish genomes (Volff, 2005). Also, a higher proportion of regulatory conserved noncoding elements (CNEs) are conserved between cartilaginous fishes and humans than between teleost fish and human suggesting that CNEs in fish are evolving faster (Venkatesh et al., 2007). This accelerated rate of evolution strongly suggests that teleost fish are indeed able to produce more novel genes than higher vertebrates.

In addition to the WGD, there are also species-specific tetraploidization and chromosomal rearrangements thought to have happened after the WGD in the teleost fish lineage that contributed to the group's genetic diversity. It has been reported that tetraploidization occurred in the rainbow trout-salmon lineage ~25 to100 Myr, and a genome compaction event in the pufferfish lineage ~ 60 to 80 Myr (Fig. 2). This was fine-tuned with the comparison of the genome sequences of zebrafish (*Danio rerio*),

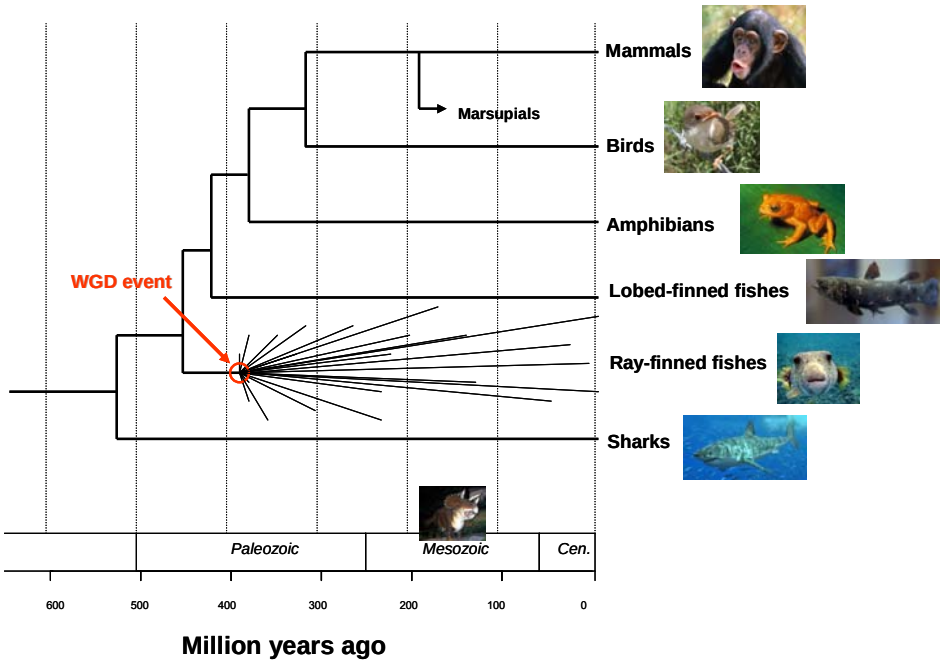

Figure 1. Timescale for vertebrate evolution. Whole genome duplication (WGD) event is indicated that is thought to contribute to the expansion and extreme diversity of ray-finned fishes. (Modified from Kumar and Hedges, 1998)

green spotted pufferfish (*Tetraodon nigroviridis*), medaka (*Oryzias latipes*) and human (Kasahara et al., 2007). It was reported that in a span of about 50 Myr after the WGD, narrowed to 336 – 404 Myr, the last common ancestor of medaka, zebrafish, and green spotted pufferfish believed to have 24 chromosomes, undergone 8 major chromosomal rearrangments (2 fissions, 4 fussions and 2 translocations). The zebrafish then diverged at about 314-332 Myr, the pufferfish, separating from medaka at about 184-198 Myr, underwent 3 fusion events, and the medaka remarkably preserving its ancestral genomic structure.

Teleost fish at the evolutionary standpoint alone is a very interesting group of species to study such that it is not surprising that there are already 5 genomic databases sequenced thus far for this taxon; the zebrafish, medaka, stickleback (*Gastoresteus aculeteus*), tiger pufferfish and the green spotted pufferfish (www.ensembl.org). From these teleost fish genome sequences, numerous gene duplications that are thought to originate from the WGD has been revealed (Stein 2007). This was clear with genes involved in signal transduction downstream of the transmembrane receptors but apparently not with

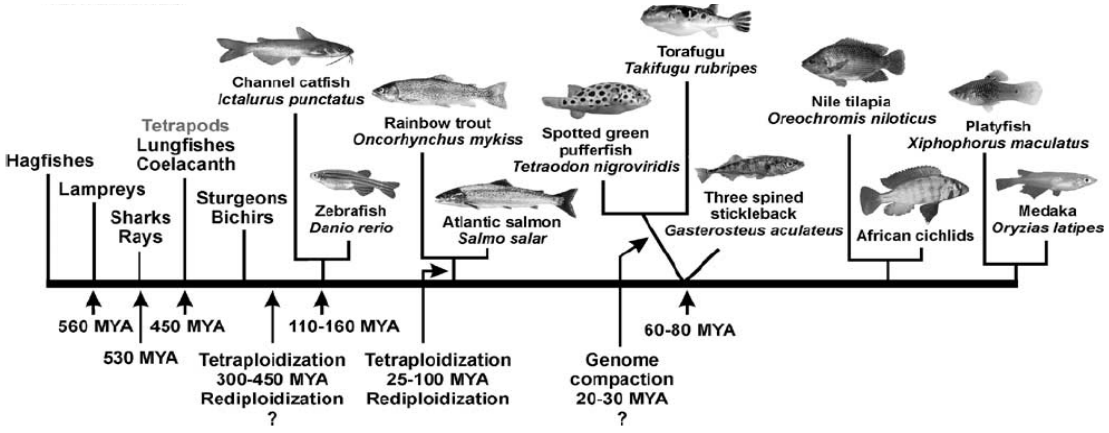

Figure 2. Genome evolution and biodiversity of fish. Tetraploidization (Whole genome duplication), rediploidization, and genome compaction that are thought to have occurred are indicated. (Adapted from Volff, 2005).

molecules involved in interactions with pathogen components. A number of these duplicated genes, mostly ATP binding proteins and transcription factor, showed that one of the paralogs are under positive Darwinian selection with significantly higher rate of molecular evolution whereas the other copy retained the original rate of evolution and did not undergo adaptive changes (Steinke et al., 2006). Again, this significant increase in evolutionary rates in one of the duplicates could be evidence of gain in novel gene function hence the diversity of teleost fish processes and species.

3. Teleost fish immune system

Teleosts or bony fishes are one of the 'lowest' vertebrate groups found to exhibit both an innate (nonspecific) and an acquired (specific) immune system (Magor and Magor, 2001). As with other vertebrates, these defense mechanisms protect the fish against pathogenic infection and re-infection.

3.1. Innate immunity

Innate immunity is the first line of defense against infection and is regarded as the primeval and hence the universal form of host defense (Janeway and Medzitov 2002). It exists in animals (vertebrates and invertebrates) and plants, and recent data suggests that it is a product of convergent rather than divergent evolution (Ausubel 2005). In fish, as in higher vertebrates, it is characterized by 3 important features (Ellis, 2001). Firstly, it is nonspecific i.e. pathogens are recognized not by their specific molecular structure but by their pathogen-associated molecular patterns or PAMPs such as the lipopolysacharride (LPS) in bacteria and double strand RNA in viruses (Medzhitov and Janeway, 1997). Secondly, it responds relatively fast (about 1 to 2 days) that includes the inducible inflammatory responses. Thirdly, it appears to be temperature-independent to function well. This nonspecific immunity is generally subdivided into two types, the cellular and humoral defense responses (Iwama, 1996). Ellis (2001) classified it further into integumental innate defenses, systemic innate humoral defenses and systemic innate cellular defenses for bacterial infection, and constitutive and responsive defenses for viral infection.

Cellular responses include the physical barrier such as mucus and epithelial tissues lining the skin, gills and stomach, which keeps infectious microorganisms from entering the body, and specialized cells (like monocytes/ macrophages, granulocytes and nonspecific cytotoxic cells) capable of killing and digesting pathogens if the latter breaches the physical barriers. These phagocytotic cells, mainly monocytes/ macrophages, granulocytes and nonspecific cytotoxic cells (NCCs) are recruited in the area of inflammation or infection from the blood, kidney and secondary lymphoid tissues by "signal molecules" or pro-inflammatory proteins several of which have been identified and partially or extensively studied in fish including chemokines, tumor necrosis factor (TNF), interleukins, transforming growth factors (TGFs) and interferons (Ellis, 2001; Magor and Magor, 2001; Secombes et al., 2001, Iwama and Nakanishi, 1996).

Humoral responses, on the other hand, employ a variety of proteins and glycoproteins capable of destroying or inhibiting growth of infectious microorganisms. These substances are present all throughout the body of the fish, from the mucus and

integument to the plasma and tissues. Anti-bacterial peptides (e.g. cecropin and pleurocidin), proteases (e.g. Trypsin-like proteases and cathepsin L and B), lectins (hemagglutinin), lysozymes, complement, pentraxins (e.g. C-reactive proteins (CRP) and serum amyloid protein (SAP)), bacterial growth inhibitor transferrins and, the anti-viral interferons and Mx proteins are some of the humoral response-related substances discovered so far (Ellis, 2001; Magor and Magor, 2001; Secombes et al., 2001, Iwama and Nakanishi, 1996). Other enzymes which could also have nonspecific defense properties or potential inflammatory functions but are less studied include hemolysin, proteinase, α2-Macroglobulin, Chitinase and α-Precipitin (Iwama and Nakanishi, 1996), prostaglandin-producing cyclooxygenase and inducible nitric oxide synthase responsible for generating nitric oxide (Secombes et al., 2001).

At the molecular level, innate immunity in higher vertebrates is triggered by the recognition of conserved microbial products by receptors generally called pathogen recognition receptors or pattern recognition receptors (PRRs) (Fig. 3). These molecules can distinguish 'infectious nonself' from 'noninfectious' self. PRRs, which include both extracellular and cytosolic recognition, mediate several pathways that give rise to the production of inflammatory cytokines and interferons (Kim and Lee 2007). In teleost fish, such mechanisms are believed to be conserved mainly because of the presence of PRR and cytokine orthologs (Stein *et al.* 2007; Plouffe *et al.* 2005). Furthermore, microarray analysis has revealed transcriptional modulation of various cloned fish PRRs and cytokines following bacterial agents (Kurobe et al. 2005, Gerwick *et al*. 2007; Peatman *et al*. 2007; Peatman *et al*. 2008). On the other hand, increasing evidence also shows that there are molecules and mechanisms that are specific to teleost fish (Stein *et al.* 2007; Plouffe *et al.* 2005).

The innate immune system, not only functions independently but may also constitute significant influence to the acquired immune system. Increasing evidence has shown that the processes of the two systems are interrelated. Dixon and Stet (2001) proposed such relationships by reviewing for example roles of Non-classical MHC class I receptors in

NK cell regulation. Van den Berg et al. (2004) also suggested that there is an evolutionary and functional link between innate and adaptive immune receptors.

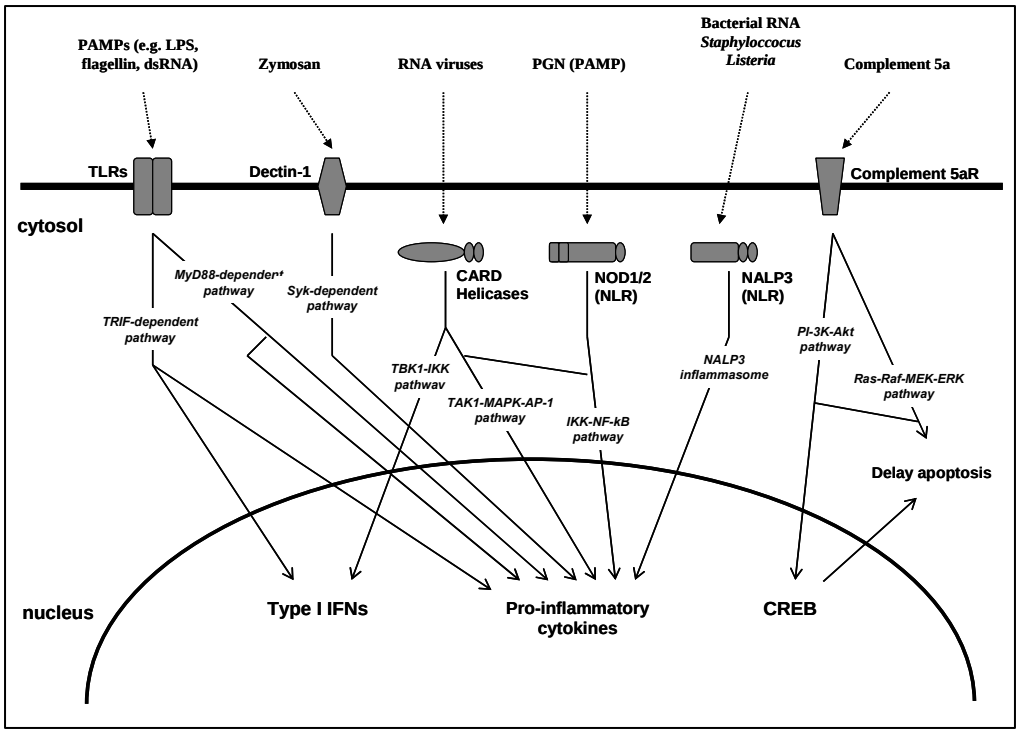

Figure 3. PAMPs, their respective PRRs and associated signaling pathways and products (Modified from Lee and Kim, 2007).

3.2. Adaptive immunity

Unlike innate immunity, the adaptive response is believed to have only appeared sometime early during the evolution of the jawed vertebrates (probably a placoderm) brought about by the invasion of transposable element into an immunoglobulin (Ig) superfamily gene, which in turn initiated the mechanism to somatically generate diversity in antigen-receptor genes (Bernstein et al., 1996; Argawal et al., 1998). The adaptive immunity is defined as having the following general components: clonally distributed antigen receptors (immunoglobulins, Igs and T cell receptors, TCRs), recombination-activating gene (RAG)-mediated gene rearrangement, primary and secondary lymphoid tissues, major histocompatibility complex (MHC)-encoded class I and class II molecules,

and somatic hypermutation, which is activation-induced cytidine deaminase (AID)-dependent (Flajnik et al., 2003). These molecules are mainly produced by B cells, T cells, NK cells and myeloid cells (Miller et al., 1998).

In teleost fish, such adaptive immune components are well conserved but at the same time show some differences, in particular the Igs and TCRs. Igs have been known to be part of the fish humoral immune system secreted to neutralize antigens in a specific manner and likewise induce the activation of the complement cascade (Rijkers, 1982). Two Igs have been described in teleost fish, IgM and IgD, since the early late 90's (Pilstrom and Bengten, 1996; Wilson et al., 1997). Recently, however, other forms of fish immunoglobulins have been identified including IgT in rainbow trout, *Oncorhynchus mykiss* (Hansen et al., 2005) and IgZ in zebrafish, *Danio rerio* (Danilova et al., 2005). Teleost IgM exists as membrane bound or secreted forms consisting of 2 subunits, the heavy chain (μ) and the Light chain (L) at ~ 70kDa and ~25 kDa, respectively. Teleost fish IgMs shows diversity in secreted "redox" forms (Kaattari et al., 1988). This has been thought to be as a result of differing disulfide bonding between adjacent heavy chains plus the presence of non-covalent bonding, which is being used by teleost fish to generate antibody diversity. On the other hand, TCRs, composed 4 subunits – TCR α, β, γ, and δ, have already been identified in various species of teleost fish (Partula et al., 1995, 1996; Hordvik et al., 1996; Wilson et al., 1998; Haire et al., 2000; Wermenstam and Pilstrom, 2001; Nam et al., 2003; Hordvik et al., 2004). The teleost fish TCR genes are organized in translocon type clusters (Zhou et al., 2003) and the α and β subunits, like in mammals, are found in the same locus of the genome and appears to have little diversity, at least in the pufferfish, as compared to mammals (Fischer et al., 2002). Very recently, however, it has been reported that the Atlantic salmon TCR α/δ genes have enormous diversity in their capacity for antigen recognition, more than in mammals, due to its large number of V and J segments, allelic polymorphisms, extensive recombinational possibilities and N-region diversity (Yazawa et al., 2008).

4. Teleost fish hematopoietic system

Because of the apparent limitations in their adaptive response, teleost fish rely heavily on innate immune mechanisms to deal with pathogens, in particular the immune-related cells of the hematopoietic or blood system. Based on early histological examinations, teleost fish have been found to be composed of different blood cell types, including nucleated erythrocytes, reticulocytes, thrombocytes, lymphocytes, fine and coarse granlocytes, and granular anucleate bodies (Catton, 1951). Recently, it was reported that the fish innate immune have nonspecific cellular responses that include monocytes/macrophages, granulocytes (neutrophils and eosinophils) (Iwama and Nakanishi, 1996). This has been supported by the characterization of granulocytes (neutrophils or sometimes called heterophils) and macrophages in zebrafish (Lieschke et al., 2001; Bennet et al., 2001). Because of cellular and morphological differences, however, blood cells between mammals and teleost fish, has always been suggested to be functionally different (Rowley *et al*. 1988). To date, there have been several novel types of cells with immune function found in teleost fish but not in mammals.

Non-specific cytotoxic cells are novel cytotoxic cell poulations reported in channel catfish, raibow trout, carop, damselfish and tilapia (Evans et al., 1984a; Evans et al., 1984b; Evans et al., 1984c; Faisal et al., 1989; Greenlee et al., 1991; McKinney et al., 1994). They have the ability to lyse various transformed human and mouse cell lines. They have been also implicated in immune resposnses against protozoan parasites (Graves et al., 1985). NCCs are found in the kidney rather than the blood. They are morpholocally similar to mammalian NK cells and also have functional similarities suggesting that they are the evolutionary precursor of NK cells.

Another type of immune-related cells in fish that have been discovered are the NK-like cells. They share functional and morphological similarities to mammalian NK cells but are distinct from NCCs (Shen et al., 2004). Furthermore they have been ruled out as B or T cells because they do not express IgM or T-cell receptors (TCR) –α, -β, -γ, nor they are neutrophils or macrophages as they do not stain with Sudan Black B and are not specific

exterase-negative. These cells exhibited cytotoxity toward allogenic targets (Shen et al., 2004).

Primitive macrophage lineage from zebrafish was shown to give rise to a number of neutrophilic granulocytes in contrast to mammalian primitive macrophage which is considered to give rise to no other cell types. Only a small fraction of these zebrafish larval neutrophils phagocytose microbes as compared to the macrophages are more attracted to stressed and malformed tissues suggesting that these cells are involved in a wider role than biodefense (Guyader *et al.* 2007). The same functional adaptation has been seen in seabream professional phagocytic granulocytes, where these cells, other than immune surveillance, have a role in the reorganization of the testis during post-spawning (Chaves-Pozo *et al*. 2005).

Recently, direct evidence have shown that, unlike mammalian B cells, rainbow trout B cell populations have potent phagocytic capability i.e. following uptake of particles, these cells could induce "downstream" degradation pathways leading to formation of phagolysosome and intracellular killing of microbes (Li *et al*. 2006).

5. Molecular mechanisms of immuno-hematopoeitc system

The formation of blood cells involved in immune system in higher vertebrates is known to be influenced by several genes and molecules. Several of these genes were found to have orthologs in teleost fish suggesting that in general, mammalian and teleost fish immuno-hematopoietic molecular processes are conserved (Fig. 4) (Song et al., 2004; Thisse and Zon, 2002). However, like the teleost-specific blood cells, there have also been teleost-specific genes that control hematopoieis, clearly suggesting that the teleost fish immuno-hematopoietic system is likewise quite different from higher vertebrates. Among these immuno-hematopoietic genes, which have been poorly understood in teleost fish, are the cytokines and their cognate receptors.

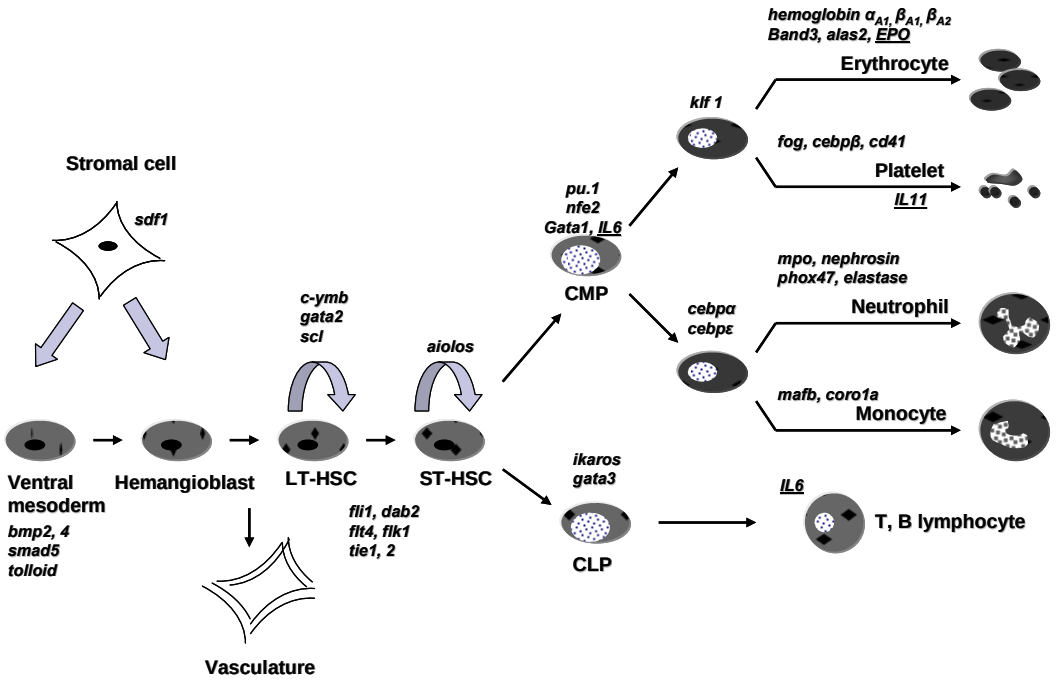

Figure 4. Reported genetic programs in zebrafish that are thought to be conserved in higher vertebrates (Modified from Song et al., 2004). Recently reported hematopoietic cytokines have been included in the figure.

5.1 Hematopoietic Cytokines

Cytokines in higher vertebrates have pleiotropic (multiple), overlapping and sometimes contradictory functions such that their classification is oftentimes difficult. These molecules have been grouped based on general names (e.g. lymphokines, monokines, chemokines, interleukins, interferons, tumor necrosis factors, colony stimulating factors etc); on functions (e.g. pro- and anti-inflammatory or innate and adaptive immunity-related); on structure (e.g. short and long chain cytokines); and on receptors used (immunoglobulin superfamily, hematopoetic growth factor (class 1-family) , interferon family (class 2- family), tumor necrosis factor (type 3-family) and chemokine receptors (7 transmembrane helix family) (Table 1). Cytokine Class 1 and Cytokine Class 2 are considered hematopoietic cytokines because of their general involvement in blood cell formation and development. Class 1 cytokines are often involved in the proliferation, recruitment to inflammation/infection sites, survival and maturation of cells (Huising *et*

al. 2006). Because of their pleiotropic nature, they can also be involved in other physiological processes like reproduction, food intake and metabolism. They share similar 4 α-helical-2 β sheet 3D structure and use the class 1 cytokine receptors (Fig. 5). Although they share little primary sequence identity, these cytokines are considered to have expanded from a single ancestor (Bazan 1990). Class II cytokines, in contrast to Class I, generally acts on minimizing damage to host after noninfectious and infectious insult, and include the interferons which mediates antiviral responses and IL10 known to be anti-inflammatory in nature (Krause and Pestka, 2005). Their structure is likewise different with Class 1 cytokines in that their β sheets have been replaced by α-helices (Langer *et al*. 2004).

Orthologous Class I and II cytokines have been identified in teleost fish (Table 1). Recent reviews also discussed the similarities of innate immune mechanisms between teleost fish and mammals where it has been establish the presence of fish orthologs for antimicrobial peptides, pro-inflammatory cytokines e.g. TNF-α, IL-1β, IL-18, interferons, chemokines and IL-8, and complement (Plouffe *et al*. 2005). Gene or protein orthology and conservation allows speculation for similarity of function but functional genomics and protein assays are desirable to ascertain function. With the whole genome sequences in fish, recent publications have started to report functionalities of immune-related genes and their proteins. An example of gene structure and function conservation between fish and humans is the action of erythropoietin (EPO) and erythropoietin receptor (EPOR) in red blood cell formation and hematopoiesis and under hypoxic conditions (Paffett-Lugassy *et al*. 2007). Another is the macrophage colony-stimulating factor (CSF-1), which like in mammals, was observed to induce proliferation of monocytes to macrophages (Hanington *et al*. 2007).

Although generally similar, differing functional attributes between teleost fish and mammalian immuno-hematopoiesis is now also being observed not only at the genetic but at the protein level as well. Th2 cytokines (IL4, IL5, IL13), the neighbor IL3 and granulocyte-macrophage colony stimulating factor (GM-CSF), which are found in a cluster in mammals, are apparently missing in teleost fish (Huising *et al*. 2006). Recently,

there have been reports of members of the IL6-ctokine subfamily that appear to only be found in teleosts. A novel class-1 helical cytokine M17 (named after the original clone number of the carp leukocyte cDNA library), cloned in 2003, was shown to be induced by alginate by and later shown to be induced by nitric oxide and can stimulate proliferation of monocytes to macrophages (Hanington and Belosevic 2007). Its apparent paralog, named M17 homologue (MSH) have been cloned recently and was shown to be induced by bacterial agents, lipopolysaccharide, peptidoglycan and the dsDNA, viral mimic polyI:C (Hwang *et al*. 2007). Another recently published fish-specific report showed that goldfish possesses a soluble macrophage colony stimulating factor receptor (CSF1R) not seen in higher vetebrates, which can inhibit CSF1-induced proliferation of monocytes into macrophages and monocyte-like cells (Hanington *et al*. 2007).

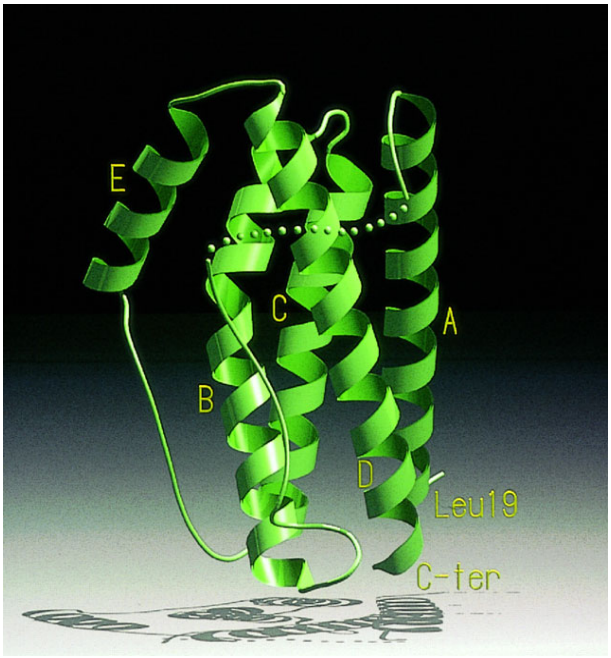

Figure 5. Ribbon representation of the IL-6 crytal structure. The four main helices are labled A, B, C and D. The extra helix in the final loop is labeled E. The missing part of the first cross-over connection is indicated by dashed line. Figure was created using MOLSCRIPT and RAYSHADE. (Adapted from Somers et al., 1997)

Table 1. Cytokines identified in mammals and teleost fish

Family	Function/Structure	Taxon	Selected members	Source
Cytokine Class 1	Involved in expansion and differentiation of cells. Have a 4-α helix bundle structure	Mammals	IL6, GCSF, IL11, IL12p35, IL23p19, IL27p28, LIF , OSM, CNTF, CLC, CT-1, CT-2 NP, Leptin, Epo, PRL, GH	Bazan 1990; Huising *et al*. 2006 Santos *et al*. 2006 Hwang *et al*. 2007
		Teleost Fish	IL6-a and -b, IL11-a and –b*, GCSF-a and –b*, Leptin, Epo, PRL, GH, M17* and M17 Homologue (MSH)*	
Cytokine Class 2	Involved in minimizing damage to host after insult. Contain more than 4-α helices.	Mammals	Type I IFN (IFNα2, IFNα4, IFNβ, IFNω1, IFNλ1), Type II IFN, IFN-γ, IL10, IL28-like, IL19, IL20, IL22, IL24, IL26	Krause and Pestka 2005; Robertsen 2006
		Teleost Fish	Type I IFN (IFNα-1, IFNα-2), IFN-γ, IL10, IL20, IL24	
Chemokines	Regulate cell migration under both inflammatory and homeostasis. Small proteins with 4 conserved Cys residues	Mammals	CXC (IL8, PF4, PBP, NAP-2), CC (MIP-1α, MIP-1β, MCP-1, MCP-2, MCP-3, RANTES); C (Lymphotactin)	Bacon *et al*. 2003; Peatman and Liu 2007 Kim *et al*. 2007
		Teleost Fish	CXC (CXC8-like, CXC-10, -12, -13, -14) CC (CCL19/21/25, CCL20, CCL27/28, CCL17/22, MIP, MCP)	
TNF Super Family	Involved in inflammation and lymphoid organ development. Compact trimers as membrane bound or soluble proteins	Mammals	TNF-α, lymphotocxin-α (TNF-β) and lymphotoxin- β	Gruss 1996; Kono *et al*. 2006
		Teleost Fish	TNF-α1, TNF-α2, TNF-N, lymphotoxin- β1 and - β1	
Interleukin 1 Family	Involved in pro-inflammatory responses. Fold rich in β-strands	Mammals	IL1-α, IL1-β, IL18	Huising *et al*. 2004
		Teleost Fish	IL1-α, IL1-β, IL18	

5.2. Hematopoietin Cytokine Receptors

In mammals, cytokine receptors are generally classified into the hematopoietins, chemokine receptors, TNF family receptors and the IL1 F receptors. Among these, the hematopoietins is the largest which mediates cytokine signals by specific binding forming a functional cytokine receptor complex (Kishimote et al., 1994). They lack cytokines following bacterial agents (Kurobe et al. 2005, Gerwick *et al*. 2007; Peatman *et al*. 2007; Peatman *et al*. 2008). On the other hand, increasing evidence also shows that they function as transcription factors to regulate gene expression (Fig. 6) (Scheller et al., 2006; Ward et al., 2000).

Hematopoietin receptors possess a conserved extracellular region, known as the cytokine binding domain (CBD), along with a range of other structural modules, including extracellular immunoglobulins (Ig)-like and fibronectin type III (FBNIII)-like domains, a transmembrane domain, and intracellular homology domains. They are divided into 2 classes depending on its CBD; the Class I receptors and the Class II receptors.

The Class I receptors are characterized by 2 pairs of conserved cysteine residues linked via disulfide bonds and a C-terminal-WSXWS motif within the CBD (Bazan, 1990). This class is further subdivided into IL-2R family, IL-3R family, the homomeric receptors and the IL-6R family, each of which consists of at least one signal transducing receptor chain containing membrane-proximal Box 1 and Box 2 motifs associated with Janus kinase (Jak) docking. IL-2Rs are primarily involved in the growth and maturation of lymphoid cells in particular proliferation, development and homeostatis of T cells, promote T helper 2 (Th2) cell development, and proliferation of natural killer (NK) cells (Gaffen et al., 2001; Fry and Mackall, 2005; Parish-Novak et al., 2000). Members of the family include IL-2R, IL-4Rα, IL-5Rα, IL-7Rα IL, IL-15Rα and associates with Jak1, Jak3 primarily activating STAT5, although certain family members can also activate STAT1, STAT3 or STAT6. IL-3Rs shares the common signal transducer chain IL-3Rβc in combination with specific chains (Boulay et al., 2003). They are associated with Jak2 and signals primarily via STAT5, although activation of other STATs has been observed

in certain cell lines, and primarily involves in the production of myelomonocytic cells e.g. differentiation of pluripotent stem cells into various myeloid progenitor cells while IL-5 is involved in eosinophil development (Mangi and Newland, 1999; Roboz and Rafii, 1999). The homomeric receptors include the erythropoietin receptor (EPOR), thrombopoietin receptor (TPOR), prolactin receptor (PRLR), and growth hormone receptor (GHR). These receptors form homodimers in the presence of their respective ligands and associate exclusively with Jak2 and signal via STAT5. EPOR and TPOR are mediators of erythroid and platelet production, respectively, GHR mediates growth and sexual dimorphism while PRLR is involved in mammary development and lactation (Richmond et al., 2005; Fishley and Alexander, 2004; Heldin, 1995; Boulay et al., 2003; Frank, 2001; Bole-Feysot et al., 1998). IL6-R family members share the subunit

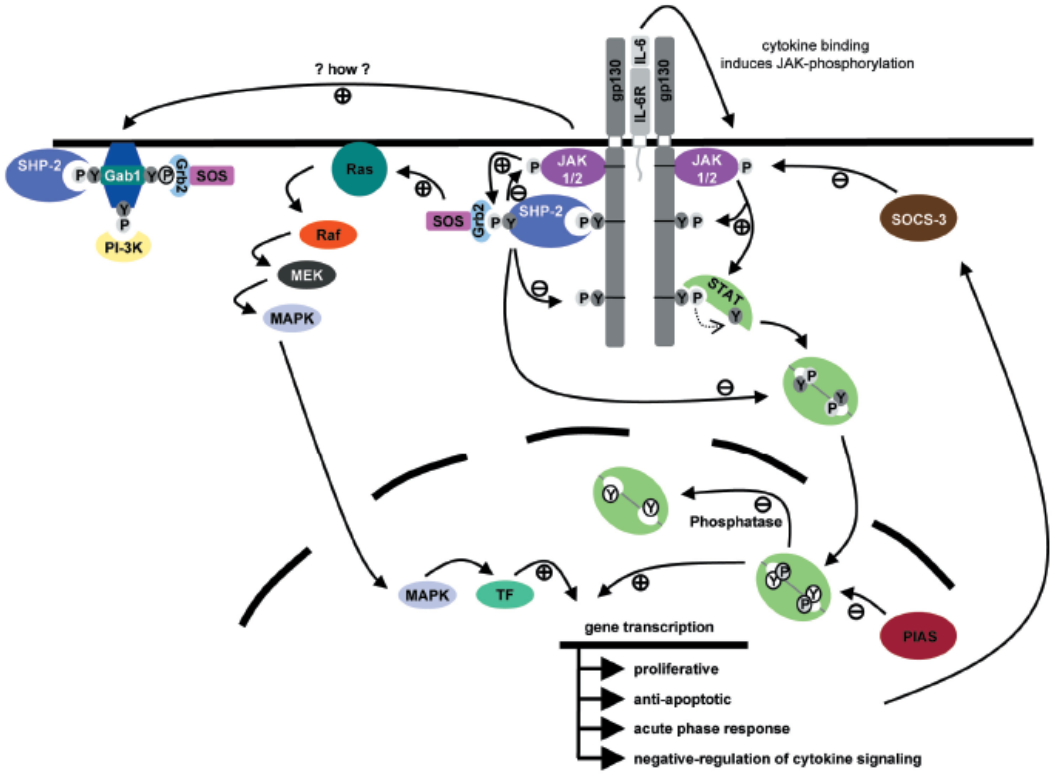

Figure 6. Example of hematopoietin receptor mediated signaling: Schematic illustration of the IL-6 signaling pathway (Adapted from Scheller et al., 2006)

glycoprotein 130 (GP130), with many also using the leukemia inhibitory factor receptor chain (LIFR). They are very critical in physiological processes such that when knockout in mice, the consequence is lethal (Yoshida et al., 1996). They generally associate Jak1,

Jak2 and tyrosine kinase 2 (Tyk2) and activates STAT1, STAT3 and STAT 5 (Heinrich et al., 1998). The members includes obesity gene receptor (OBR), granulocyte colony stimulating factor (CSF3), IL-6R, IL-11R, IL-27R, ciliary neutrophic factor receptor I and II (CNTF-I and -II R), CTF1R, LIFR, Oncostatin M receptor I and II (OSMR-I and –II), GP130-like molecule receptor (GLMR), IL-12R and IL23R. Not all of the members utilize GP130. The subfamily IL-12R consists of complexes containing the shared receptor, IL-12p40 and IL-12Rβ1, along with the specific IL-12Rβ2 or IL-23RRα and they activate a more specific downstream component like IL-12R activates STAT4 and IL-23R activates STAT3 (Watford et al., 2004). On the other hand, CSF3R and OBR form homodimers but activate the same Jaks and STATs as GP130 (Devos et al., 1997; Hiraoka et al., 1994). These IL-6R mediates general and specific functions; CNTFR promotes survival and differentiation of cells within the nervous system, IL-6R mediates immune, hematopoietic, and thrombopoietic responses, L-12R subfamily functions in innate immunity, GCSFR plays a key role in granulocytic development and OBR is involved in appetite control (Watford et al., 2004; Ito, 2003; Elson et al., 2000; Lieschke et al., 1994; Tartaglia et al., 1995).

Class II receptors also have 2 pairs of cysteine but with a different arrangement to Class I and also lack the WSXWS motif (Bazan, 1990). Dimerization entails a long intracellular ligand binding receptor and a short intracellular accessory receptor. These receptors are primarily involved in antiviral and inflammation. Members include the antiviral receptors Type I IFNR for IFNα/β/κ/ω/ε, Type II IFNR for IFNγ and IFNλR for IFNλ 1-3 (Kotenko and Langer, 2004). Type I IFNR and IFNλR have been shown to be involved in induction of antiviral state via the Jak2 and Tyk2 in a similar STAT-2 dependent downstream pathway while mice deficient in IFNγ are susceptible to pathogenic bacteria (Hwang et al., 1995). There are also the non-antiviral receptors that include the IL-10R2, IL-20R1, IL-20R2 and IL-22R1 with the 3 cytokine specific receptor subunits IFNλR1, IL-10R and IL-20R1, crating a total of 6 receptor complexes. These receptors associate via the Jak2 and Tyk2 and signals via STAT1, STAT3 and STAT5 (Kotenko and Petska, 2000). These non-antiviral receptors, except for IFNλR, modulate the inflammatory response (Renauld, 2003; Coni et al., 2003)

Teleost fish hematopoietin receptors have been identified and cloned although most of them are predictions at best from the existing genomic databases from zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), stickleback (*Gasterosteus aculeatus*), tiger pufferfish (*Takifugu rubripes*) and the green spotted pufferfish (*Tetraodon nigroviridis*) (www.ensembl.org). Some of these receptors been reported in the green spotted pufferfish genome although their orthology is not yet clear since assignment of names was done only up to the *in silico*-prediction level and the genes were compared only to human (Jaillon et al., 2004). These include Growth Hormone Receptor (GHR), Prolactin Receptor (PRLR), Erythropoietin Receptor (EPOR), Interleukin 12 Receptor γ (IL2Rγ), Interleukin 7 Receptor A (IL7Rα), Interleukin 12 Receptor β/ Interleukin 4 Receptor A (IL2Rβ/IL4RA), Interleukin 21 Receptor (IL21R), Interleukin 12 p40 (IL12p40), Ciliary Neutrophic Factor (CNTFR), Interleukin 11 Receptor A (IL11Rα), Interleukin 13 Receptor A (IL13Rα), Interleukin 6 Receptor A (IL6Rα), Thrombopoietin Receptor, Interleukin 12 Receptor β2 (IL12Rβ2), glycoprotein 130 (gp130), Leukemia Inhibiting Factor Receptor (LIFR) and Obese Protein Receptor (OBR) or Leptin Receptor (LEPR). Fish cytokine receptors that have been cloned and characterized so far include LIFR and PRLR of gold fish (Hanington and Belosevic, 2005; Tse et al., 2000) and the growth hormones of fugu, zebrafish, Southern catfish and Nile tilapia (Jiao et al., 2006). In terms of cytokine receptors, there have also been reports of such genes that do not possess clear orthology to known receptors and appears to be more of ancestral in structure. The Leukemia Inhibitory Factor Receptor (LIFR)-like molecule identified in goldfish was suggested to be the ancestral molecule for mammalian LIFR and Oncostatin M Receptor (OSMR) (Hanington and Belosevic 2005) although it still not clear whether the LIFR-like genes predicted in the chromosome 12 of the green spotted pufferfish is orthologous to the goldfish LIFR-like genes or to teleost OSMR as well (Jaillon *et al*. 2004).

6. Perspectives

6.1. Information gaps

Clearly, many of the teleost orthologs of immuno-hematopoietic- related molecules are yet to be identified. In particular, the members of the Th2 cytokines which include the granulocyte-macrophage colony-stimulating factor (CSF2) are yet to be located and are thought to be missing in this group (Huising et al., 2006). Also, as initial cloning studies and the WGD suggests, it is possible that these immuno-hematopoietic molecules are in duplicates. It is not clear, however, the fates of some of these unidentified paralogs in each of the fish species especially now that it is becoming clear that the evolution of duplicated genes in teleost fish, or its genetic diversity for that matter, is species-specific. In addition, if indeed some of these genes are in duplicates, the fate of each duplicate (nonfunctional, neofunctional, subfunctional) should be determined as this could have valuable implication to the molecular processes where they are involved, especially if both genes are functional.

Most of the studies for the teleost fish immuno-hamtopietic cytokines that includes both the class 1 (hematopoietins) and class 2 (interferons) are so far only up to cloning and expression levels (Huising et al., 2006). Many pertain to large scale evolutionary and transcriptomic profiles and very few functional studies have been done. Initial functional studies indicate that there abound cellular and molecular mechanisms unique only to teleost fish that needs to be studied, which can prove to yield novel and important results.

6.2. Useful tools

The tools available to teleost fish like fish cell lines and specific antibodies to serve as cell markers are very limited and thus needs to be developed. Only through these tools that specific functional attributes can be clearly analyzed. Also, there is a need to do protein analysis as most of publications are up to the transcriptomic levels. The development of efficient and fish-specific systems to produce biologically active recombinant fish proteins could therefore prove to be very valuable.

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CHAPTER 2

Molecular characterization of immuno-hematopoietic cytokines from teleost fish: granulocyte colony-stimulating factor, M17 homologue and interleukin 11b

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Molecular characterization of immuno-hematopoietic cytokines from teleost fish: granulocyte colony-stimulating factor, M17 homologue and interleukin 11b

Abstract

The innate immune system of fish consists of nonspecific cellular responses that include monocytes/macrophages, granulocytes (neutrophils and eosinophils) and nonspecific cytotoxic cells (NCCs). For these immune-related cells to develop proliferate, differentiate, survive and mature, important cytokine molecules are needed to be activated. In teleosts, some of these molecules have been identified and characterized but because of their rapidly-evolving nature, many of these are yet to be discovered in spite of the available fish genomic resources. Here we cloned and characterized 3 immuno-hematopoietic cytokines in Japanese flounder, *Paralichthys olivaceus*: the Granulocyte colony-stimulating factor (CSF3), Interleukin 11 type b (IL11b) and an M17 homologue (MSH).

CSF3 is a glycoprotein cytokine which influences the hematopoiesis of the phagocytic neutrophils and its precursors, and has been used extensively in cancer therapy and for the treatment of neutropenia in mammals. We report the first CSF3 genes from 3 teleost fishes: Japanese flounder, fugu, *Takifugu rubripes* and green spotted pufferfish, *Tetraodon nigroviridis.* Paralogous fugu and pufferfish CSF3-1s and CSF3-2s are shown to be the ancestral and duplicate genes, respectively. Moreover, we demonstrate that the Japanese flounder CSF3 gene is at least involved in immunity based on its basal expression in immune-related tissues, and its up-regulation in kidney and peripheral blood leukocytes by LPS, a combination of conA/PMA and by poly I:C, a known interferon inducer.

IL-11, on the other hand, is involved in physiological processes including blood production, bone formation and placentation. The IL-11 paralogues (IL11a and IL11b) have been identified in fish with only IL11a from carp and trout have been characterized and analyzed for its expression thus far. Japanese flounder IL11b, *po*IL11b is confirmed as such by structural and phylogenetic analysis. *po*IL11b doesn't show constitutive expression in tissues of adult fish except for the very slight expression in kidney and spleen, and the very high expression in peripheral blood leukocytes (PBLs). *po*IL11b is transiently up-regulated by bacterial LPS and increasingly stimulated by the IFN inducer poly I:C in kidney, spleen and peripheral blood leukocytes (PBLs) of adult fish *in vitro*. In addition, it is very slightly stimulated by *Edwardsiella tarda* infection but is highly expressed after hirame rhabdovirus (HIRRV) infection in kidney of juvenile fish, suggesting that *po*IL11b, aside from its role in bacterial infection, is well involved in antiviral responses.

Finally, we studied a Japanese flounder MSH, which is closely associated with the IL6 subfamily member M17. MSH had significant identity but exhibited contrasting

expression with fish M17s. Subsequent *in silico* search and full annotation of the M17 orthologue in zebrafish, *Danio rerio*, MSH orthologues in tiger puffer, green spotted pufferfish (*Tetraodon nigroviridis*) and stickleback (*Gastorosteus aculeatus*), as well as structural, synteny comparisons and phylogenetic analysis with known IL6-cytokines, we determined the novelty of the fish MSH. Japanese flounder MSH was observed to be highly expressed in immune-related tissues and are induced by immune stimulants, LPS, poly I:C and PG *in vitro* suggesting that it is involved in fish immunity particularly against viral and bacterial agents, a functional feature exhibited by previously reported fish cytokines.

1. Introduction

The IL6-cytokine subfamily activate target genes involved in cell differentiation, survival, apoptosis and proliferation, have pro- and anti-inflammatory properties and are major players in hematopoiesis, as well as in acute-phase and immune responses. These cytokines have the same four α-helix bundle motif and share a common signal transducing receptor component, glycoprotein 130 (gp130) (Heinrich et al., 2003; Huising et al., 2006). They include interleukin 6 (IL6), interleukin 11 (IL11), leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1), cardiotrophin-2 (CT-2) and ciliary neurotrophic factor (CNTF). While granulocyte colony-stimulating factor (CSF3) is not classified as an IL6 cytokine because it doesn't utilize gp130, its protein domain is well conserved with IL6 forming the SCOP IL6/CSF3/MGF protein family (Santos et al., 2006). IL6 is a pleiotropic cytokine involved in numerous biological functions e.g. oncogenesis, inflammation, immune regulation and hematopoiesis (Naka et al., 2002). IL11 is a multifunctional cytokine that stimulates hematopoietic progenitor cells and exerts a series of important immunomodulatory effects (Kawashima et al., 1992). LIF is a pleiotropic cytokine based on its growth and differentiation activities on haematopoietic cells (Escary et al., 1993). OSM is multifunctional cytokine produced by activated T lymphocytes and monocytes and shares properties with all the members of this family of proteins (Tamura et al., 2002). OSM is structurally and functionally very similar to LIF, which suggests that the two genes arose by duplication (Tamura et al., 2002; Rose et al., 1993). CSF3 influences neutrophil life cycle in mammals (Barreda et al., 2004).

CSF3, a glycoprotein cytokine, mediates the proliferation, survival, terminal maturation and functional activation of mammalian neutrophils and its precursors during inflammation or steady-state in a lineage-specific manner (for review, see Basu et al. 2002). CSF3 is one of the few cytokines used successfully as recombinant therapeutics in mammals (Vilcek and Feldmann 2004; Welte et al. 1996).They have been reported to be produced by different kinds of cells including monocytes/macrophages and lymphocytes (Sallerfors, 1994), fibroblasts (Kaushansky et al., 1988), endothelial cells (Zsebo et al., 1988), astrocytes (Aloisi et al., 1992), bone marrow stromal cells (Fibbe et al., 1988a), T-lymphocytes (Ichinose et al., 1990) and polymorphonuclear granulocytes (Lindemann et al., 1989). The increase in production of CSF3 is very sharp and abrupt (having a half life of 4-10 h in circulation and 1-2 days in tissues) in response to endotoxins or secondary mediators such as tumor necrosis factor (TNF), Interleukin-1 and interferon-γ (IFN-γ), suggesting that it is a vital regulator of granulocyte production during inflammation and immune responses (Demetri and Griffin, 1991). CSF3 could also be induced by PHA and PMA (Oster et al., 1989a), IL-3 (Oster et al., 1989b), IL-4 (Wieser et al., 1989), granulocyte-macrophage colony-stimulating factor (CSF2) (Sallerfors and Olofsson, 1991; Oster et al., 1989b) and macrophage colony-stimulating factor (CSF1) (Ishizaka et al., 1986). However, CSF3 as well the other colony stimulating factors, are yet to be identified and characterized in lower vertebrates.

IL11 is a pleiotropic cytokine that possesses many functions such as production of thrombocytes (thrombocytopoiesis), megarkayocytes (megakayo-cytopoiesis) and other blood cells, bone formation and osteoblastosis, and in placental development (for reviews see Huising et al., 2006; Du and Williams, 1997) . Because of its influence in hematopoiesis, particularly in megakaryocyte and thrombocyte development, it has been used to prevent thrombocyte/ platelet loss following cancer therapy (Kurzrock, 2005) and is also being explored as a cure for neonatal thrombocytopenia (Ramasethu, 2004). Mammalian IL11 is a single copy signal molecule that is expressed by and acts on various types of cells. It is composed of a high number of proline (P), leucine (L) and positively charged amino acids making it a basic molecule. It possesses a 4 α-helix bundle structure even in the absence of disulphide binding cysteine (C) residues, made

possible by hydrophobic interactions (Czupryn et al., 1995). Because of the 4 α-helix configuration and the formation of a hexameric complex by IL11, IL 11 receptor and gp130 during signal transduction (Heinrich et al., 2003), IL11 has been classified as a member of the class-1 helical cytokine. Teleost fish IL11 orthologue was first reported in trout (Wang et al., 2005). Subsequent cloning studies on carp coupled with *in silico* analysis of zebrafish (*Danio rerio*), tiger pufferfish (*Takifugu rubripes*) and green spotted pufferfish (*Tetraodon nigroviridis*) genomes allowed for the identification of a duplicated fish IL11 gene (IL11a and IL11b) (Huising et al., 2005). In both these reports, a partial EST fragment (AU090873) from Japanese flounder has been included in the phylogenetic analysis and was shown to cluster with fish IL11b. The IL11a and IL11b genes from carp, trout, zebrafish, tiger pufferfish and green spotted pufferfish have been thoroughly characterized for its genomic and secondary protein structures. However, only IL11a from carp and trout has been so far investigated for its expression where it was found to be ubiquitously expressed in all tissues including brain, intestine, skin, muscle, liver, spleen, head kidney, kidney, thymus and gills except PBLs. IL11a expression has likewise been observed to be enhanced by lipopolysaccharide (LPS), bacteria (*Aeromonas salmonicida* MT423), conA, poly I:C and recombinant IL-1β, albeit at different levels, but significantly inhibited by cortisol (Wang et al., 2005; Huising et al., 2005) . It is not known whether IL11b is functional and to what extent is its role in fish immunity in relation to a functional duplicate IL11a given that there is only a single IL11 protein in higher vertebrates.

M17 is a member of the IL6-cytokine subfamily have been identified and characterized only in teleost fish, in carp (*Cyprinus carpio*) and in goldfish (*Carassius auratus*) (Fujiki et al., 2003; Hannington et al., 2007). Carp M17 is predominantly expressed in the brain and to some extent in peripheral blood leukocyte while goldfish M17 was observed to induce goldfish macrophage differentiation and nitric oxide production, has been reported.

Japanese flounder is one of the economically important culture species that is being impacted by diseases such as *Edwardsiella tarda* and HIRRV (Oh and Choi, 1998; Plumb,

1999). As such, it has been the subject of extensive studies on host-pathogen interactions and immunology at the molecular level.

In this study, we established the presence of CSF3 genes from 3 teleost species (Japanese flounder, fugu, green spotted pufferfish, as well as present evolutionary characteristics of CSF3 genes, as well as its constitutive and immuno-stimulated expression. We also analyzed the constitutive expression of the paralogous CSF3s in fugu (*tr*CSF3-1 and *tr*CSF3-2) and pufferfish (*tn*CSF3-1 and *tn*CSF3-2). We also report the cloning of the full Japanese flounder IL11b cDNA (*po*IL11b) and the analysis of its expression, constitutively and in response to LPS and poly I:C stimulation *in vitro,* and *E. tarda* and HIRRV infection *in vivo*. *po*IL11b amino acid structure has 2 extra cysteine residues compared with fish IL11a, which may explain the significant difference in expression patterns between the duplicate genes. *po*IL11b expression appears to be in contrast with fish IL11a suggesting a complementation of function of the 2 genes. Furthermore, *po*IL11b, assumed to be a pro-inflammatory cytokine, is interestingly more involved in antiviral rather than bacterial responses. Finally, we identified a novel cDNA in Japanese flounder that resembles M17 in general structure but not in mRNA expression in tissues, which we named M17 homologue (*po*MSH). Using *in silico* analysis and comparison to Japanese flounder MSH, we were able to fully annotate the MSH orthologues from fugu, pufferfish, and stickleback (*Gastoresteus aculeatus*) as well as the M17 orthologue from zebrafish. From this, we determined that MSH is a novel gene based on comparative structural and expression analysis. We also showed that Japanese flounder MSH is involved in fish immunity, and thus exhibits a cytokine-like function, as it is constitutively expressed in immune-related tissues/cells and is significantly induced in primary kidney cell culture by bacterial agents LPS and PG and by the interferon inducer, polyI:C.

2. Materials and Methods

2.1. Cell culture

HINAE cells were grown in Leibovitz's L-15 medium (Gibco-BRL, Grand Island, NY) supplemented with 15% FBS (JRH Bioscience, Lenexa, KS) and 100 IU ml-1 penicillin G and 100 μg ml-1 streptomycin (Gibco-BRL, Grand Island, NY).

2.2. Molecular cloning

The full-length Japanese flounder CSF3 cDNA and gene were determined following Hirono et al. (2000) with minor modifications. A 47 a.a.-long EST clone (Accession no: AU260798) showing putative homology to human CSF3 was used as a probe to screen a Japanese flounder kidney cDNA library. Subsequently, the ORF of the generated putative CSF3 cDNA, was used to screen a previously constructed Japanese flounder genomic BAC library (Katagiri et al 2000). Specific and overlapping forward and reverse primers, designed using Web primer (http://seq.yeastgenome.org/cgi-bin/web-primer) from the putative CSF3 cDNA, were used to amplify positive BAC clones. The full-length Japanese flounder IL11 type b cDNA was cloned by first selecting an EST clone (Accession no: AU090873) showing putative homology to published fish IL11. An anti-sense primer (5'-GTCCACCTGATGGATCATCG-3') was then designed from the upstream portion of this clone and, in partner with M13 reverse primer (5'-AGCGGATAACAATTTCACACAGG-3') amplified a resulting ~ 200 bp fragment using a previously constructed λZipLox vector-based Japanese flounder cDNA library (GIBCO BRL/Life technologies) as template. For M17 homolgue, a previously constructed cDNA library (Arma et al., 2002) from Japanese flounder kidney cells stimulated with ConA/PMA was screened using a cDNA fragment homologous to carp M17. Hybridization was performed as described previously (Hirono et al., 2000).

2.3. In silico analysis

CSF3s, IL11s and M17/MSH for fugu, green spotted pufferfish, stickleback and zebrafish were automatically and manually mined from the existing genome resources accessed through the *Ensembl* genome browser (http://www.ensembl.org/). cDNA sequences were used as BLAST probe, using different BLAST algorithms employed by the *Ensembl*

server. We mapped the CSF3 and MSH clusters by identifying conserved flanking genes in human and mouse, and used this cluster as marker to pinpoint the specific position of the gene in the fish genome

The nucleotide sequence, translated amino acids, isoelectric points and average molecular weight were analyzed and determined using GENETYX 7.0.3 (GENETYX Corporation). SignalP (http://www.cbs.dtu.dk/services/SignalP/) and NetNGlyc 1.0 (http://www.cbs.dtu.dk/ services/NetNGlyc/) servers were used to predict signal peptide cleavage and *N*-glycosylation sites, respectively. Identities were calculated using BLASTp (BLOSUM 62) implemented in BLAST 2 SEQUENCES (http://www.ncbi.nlm.nih.gov/ blast/bl2seq/wblast2.cgi) and the complete multiple amino acid alignments were carried out in CLUSTAL X 1.81 using default parameters. Protein domain prediction was carried out using ProDom (http://prodom.prabi.fr/prodom/current/html/home.php) and 123D+ (http://123d.ncifcrf.gov/123D+.html) servers. For phylogenetic analysis, we used the NJ algorithm (Saitou and Nei, 1987) implemented in the MEGA3 (http://www.megasoftware.net/index.html/) employing the Poisson correction method with 1000 bootstrap re-sampling and with complete deletion of gap sites. The bootstrap consensus tree was shown.

2.4. Constitutive expression in tissues

For RT-PCR analysis of constitutive expression, total RNA was extracted from brain, eyes, gills, kidney, heart, intestine, PBLs, liver, muscle, skin, spleen, stomach from 3 apparently healthy Japanese flounder. cDNA synthesis was done in each of the tissue samples. Resulting cDNA were amplified using primers in Table 1. PCR conditions were: initial denaturation at 95^{o}C for 5 min, 30 cycles (95^{o}C - 30s, 55^{o}C - 30s, 72^{o}C - 1 min), and final elongation at 72^{o}C for 5 min. The PCR products were electrophoresed on a 1.0 % agarose gel containing ethidium bromide. Resulting bands were photographed with a densitometer (Atto) and were semi-quantitatively assessed for their relative expression following Lindenstrom et al (2004) using the ImageJ software (Abramoff et al., 2004)

2.5. Expression in tissues after in vitro immunostimulation

Immunostimulation studies was carried out by treating primary cultures of kidney, spleen and PBLs, taken from about 3 kg fish samples, with final concentration of 0.5 mg/ml LPS, combination of ConA/PMA and polyI:C, and with phosphate buffer saline (PBS) as control sampled at 1, 3 and 6 hrs post-induction. Total RNA was extracted from the treated and control cells for cDNA synthesis and subsequent RT-PCR analysis using above stated primers. Primers for Japanese flounder, Mx (Caipang et al., 2003) and β-actin (Katagiri et al., 1997) genes were used for comparative analysis and as controls. PCR conditions were: initial denaturation at 95^{o}C for 5 min, 26 cycles (95^{o}C - 30s, 55^{o}C - 30s, 72^{o}C - 1 min), and final elongation at 72^{o}C for 5 min.

2.6. Expression in tissues after infection in vivo

Apparently healthy juvenile fish samples weighing about 2 grams each were used in the *in vivo* studies. Five fish individuals were placed in re-circulating aquarium tanks. For the *E. tarda* experiment, 1 tank containing 15 fish samples were infected with the bacteria (2.5 x 10^7 CFU/ml) by immersion while 1 untreated tank was used as a control. Kidney from 5 fish samples was sampled at 1 day, 3 days and 7 days post-infection and also from the control. For the HIRRV, 15 fish samples were intramuscularly injected with the virus (3.2 x 10^3 $TCID_{50}$) and another 15 with PBS for the control. Kidney from 5 samples was taken at 1 day, 3 days and 7 days post-injection. For each of the sampling time points, kidney was pooled and then total RNA was extracted for cDNA synthesis and subsequent RT-PCR analysis. *po*IL11b, CSF3, Mx and β-actin gene primers (same as above) were used for the analysis. PCR conditions were: initial denaturation at 95^{o}C for 5 min, 26 cycles (95^{o}C - 30s, 55^{o}C - 30s, 72^{o}C - 1 min), and final elongation at 72^{o}C for 5 min. For M17 homologue, head kidney was dissected from Japanese flounder and then mash-filtered using a sterile mesh net and a syringe plunger in a 24-well petri dish containing RPMI 1640 (NIPRO, Japan). This was done in 3 apparently healthy fish. The concentrations of stimulants used were: poly I:C (1 ug/ml), LPS (1 ug/ml) and PG (1 ug/ml). Cells were harvested after 0, 1, 3, and 6 hrs post-stimulation. Absolute copy

Table 1. Primers used for cloning and RT-PCR analysis

Fish species	Primer name	Primer sequence
Fugu (*Takifugu rubripes*)	trCSF3-1AF	TGAACATCCTGATTGTCCTCG (sense)
	trCSF3-1AR	ATCTGTCATCTGGTTCCTCGT (anti-sense)
	trCSF3-2AF	ACAGACATGACCGACCTGACA (sense)
	trCSF3-2AR	TCCTGTAGGTGCTGATGGCT (anti-sense)
Green spotted pufferfiish (*Tetraodon nigroviridis*)	tnCSF3-1AF	CATGCACATCCTCATTGTCCT (sense)
	tnCSF3-1AR	TTATCTGCTTTGGTCCAGGCT (anti-sense)
	tnCSF3-2AF	ACGGACAGACATGATCCACCT (sense)
	tnCSF3-2AR	TGCTGCTCCTGTAGCTGTTGA (anti-sense)
Japanese flounder (*Paralichthys olivaceus*)	poCSF3-2F1	CAGAGGGACAGACAGACATG (sense)
	poCSF3-F335	CGCCACCCCCATCCTCAAACCA (sense)
	poCSF3-Rev88	TCAGAGTCCATGTCGTCTG (anti-sense)
	poCSF3-5RACE	GGTCACCATCATCTGCAGGT (ani-sense)
	poCSF3-R435	AACATGGTCCCCGACGACCCTC (anti-sense)
	poCSF3-2R	ACGTGCGATTCGTCAATGGC (anti-sense)
	poIL11R	GTCCACCTGATGGATCATCG (ani-sense)
	poIL11F1	CACTGGAGTCAGAGGAGGTC (sense)
	poIL11R1	TGACTCTCCTGCCTCCAGAG (anti-sense)
	poM17F1	TCCGATTTGCCTGAGATACC (sense)
	poM17R1	TGGGAAGAGGCTCTGGTAGA (anti-sense)
	poM17RTF1	CGTCTTCCAACAGAAGGTCT (sense)
	poM17RTR1	AAGGTGGCTGGTACATCAAT (anti-sense)
	poM17RTF2	TGCGGTCCTAAAGACCCATT (sense)
	poM17RTR2	AAGAGCAAAGTCTGCAGGGG (anti-sense)

number of the Japanese flounder M17 following immunostimulation of head kidney was determined using Real-time PCR. Specific primer sets used are in Table 1 as well as beta-actin primers (Katagiri et al, 1997). The Real-time PCR assay was carried out with the SYBR Green PCR master mix (PE Biosystems) following the manufacturer's protocol.

3. Results

3.1. Japanese flounder CSF3

We isolated a putative Japanese flounder CSF3 cDNA and gene (Fig. 1) with a decided ORF of 633 bp, encoding for a 211 putative amino acid residues, having a predicted molecular weight of about 21 kDa and a computed signal peptide cleavage site between Ser_{21} and Val_{22}. It has a 5 exon- 4 intron gene configuration as confirmed by the splice donor (cag) and acceptor (gt) sequences. There were numerous and readily observable regulatory elements in the UTRs including the transcription-important "TATA" box in

the 5' region, and the polyadenylation signal "AATAAA" and numerous conserved AU-rich elements (AREs) in the 3' area.

Figure 1. Complete CSF3 gene sequence from Japanese flounder, Paralichthys olivaceus. Accession number is AB158644 (cDNA) and AB200968 (gene). The typical TATA signal sequence is boxed, AU-rich motif are italized and underlined. UTRs are in lower case and tandem repeats are underlined.

Using putative Japanese flounder CSF3 as a probe to *t*BLAST*n* fugu and green spotted pufferfish genomes in *Ensembl*, we obtained partially predicted CSF3 homologous genes in fugu (*SINFRUG00000157575* on Tr. Chr. Un, scaffold 1637) and in green spotted pufferfish CSF3 gene using Genoscope (*GSTENG00024099001* on Tn. Chr. 2, SCAF

1478). Taking into consideration the whole genome duplication theory in teleosts, we then searched for the duplicate copies of fugu and pufferfish putative CSF3 genes using a combination of automatic and manual BLAST, and synteny comparisons (results described below). We were able to isolate the duplicate CSF3 gene copy of the fugu (Scaffold 571, ~ position 105000; Fig. S3) and pufferfish (Scaffold 13844, ~ position 46234000; Fig. S5). We used the above procedure to find the CSF3 genes in the zebrafish genome resource but failed. The comparative characteristics of teleost fish CSF3s as well as their counterparts in chicken (MGF), mouse and human are in Table 1.

Table 2. Comparison of known and unique characteristics of human, mouse and fish CSF3 orthologues, including the chicken MGF.

CSF3 Orthologues	Gene size (kb) from start to stop codons	No. of Introns/ Exons	Open Reading Frame (ORF) size (bp)	Predicted protein size (a.a)	Predictes molecular mass (kDa)	Signal peptide length/ cleavage site	TATA box position	Kozak-type concensus sequence (A/GCCATG G)	Detected concensus CSF box in promoter (GAGRTTCC A/CC)	Observable, known AU-rich elements (AREs). *Nos. in parenthesis*	Simple sequence repeats (SSRs) or microsatellites
Human	1.49	4/5	612	204	22	30/ Ala^{30}	- 334	CCCATGG	GAGATTCCCC	AUUUA (6)	----
Mouse	1.58	4/5	624	208	22	30/ Ala^{30}	- 239	TCCATGG	GAGATTCCCC	AUUUA (5)	----
Chicken MGF	1.24	4/5	603	201	22	23/ Gly^{23}	- 344	ACCATGT	GTGAATCCCC	AUUUA (8)	----
Flounder *po*CSF3-2	2.14	4/5	633	211	23	21/ Ser^{19}	- 857	GACATGG	GAAAATCCCG	AUUUA (5)	GGGTTCTTC
Fugu *tr*CSF3-2	1.59	4/5	609	202	22	19/ Ser^{19}	- 495	GACATGA	GGAAAACCCG	AUUUA (4) UUAUUUAUU (1)	CA; GT;
Pufferfish *tn*CSF3-2	1.86	4/5	609	202	22	19/ Ser^{19}	- 798	GACATGA	GGAAAACCCG	AUUUA (5)	GT; CA; GGTTCCGTAC CGGCCT
Fugu *tr*CSF3-1	1.50	4/5	636	211	23	18/ Gly^{18}	- 393	GCCATGA	GGGAATCCCA	AUUUA (5)	GT; CT
Pufferfish *tn*CSF3-1	1.41	4/5	561	186	20	18/ Gly^{18}	- 412	GCCATGC	GGAAATCCCC	AUUUA (4)	GT; CT

A CSF3 cluster synteny map (Fig. 2) showing the CSF3 locus in human (Chromosome 17), mouse (Chromosome 11), chicken (Chromosome unknown), green spotted pufferfish (Chromosome 2 and Scaffold 13844) and fugu (Scaffolds 571 and 1637) has been constructed. From this map, we found that the CSF3 locus was conserved in humans, mouse and chicken as evidenced by the retention of the ORMDL3, GSDM, GSDML, LRRP, PSMD3, CSF3 and THRAP4 genes across the 3 species. With this we were able

to confirm and complete the identity and positions of fugu and pufferfish as well as the chicken CSF3 orthologues. The CSF3 cluster syteny map in addition to supporting orthology and paralogy of fish and chicken CSF3s, showed that the CSF3 locus in green spotted pufferfish (*tn*CSF3-1) at Chr.2 and fugu (*tr*CSF3-1) at scaffold 571 were the loci which were directly related to the mammalian and avian CSF3 locus. This relationship was clearly seen through the conservation of THRAP4 and LRRP genes, and the shifted

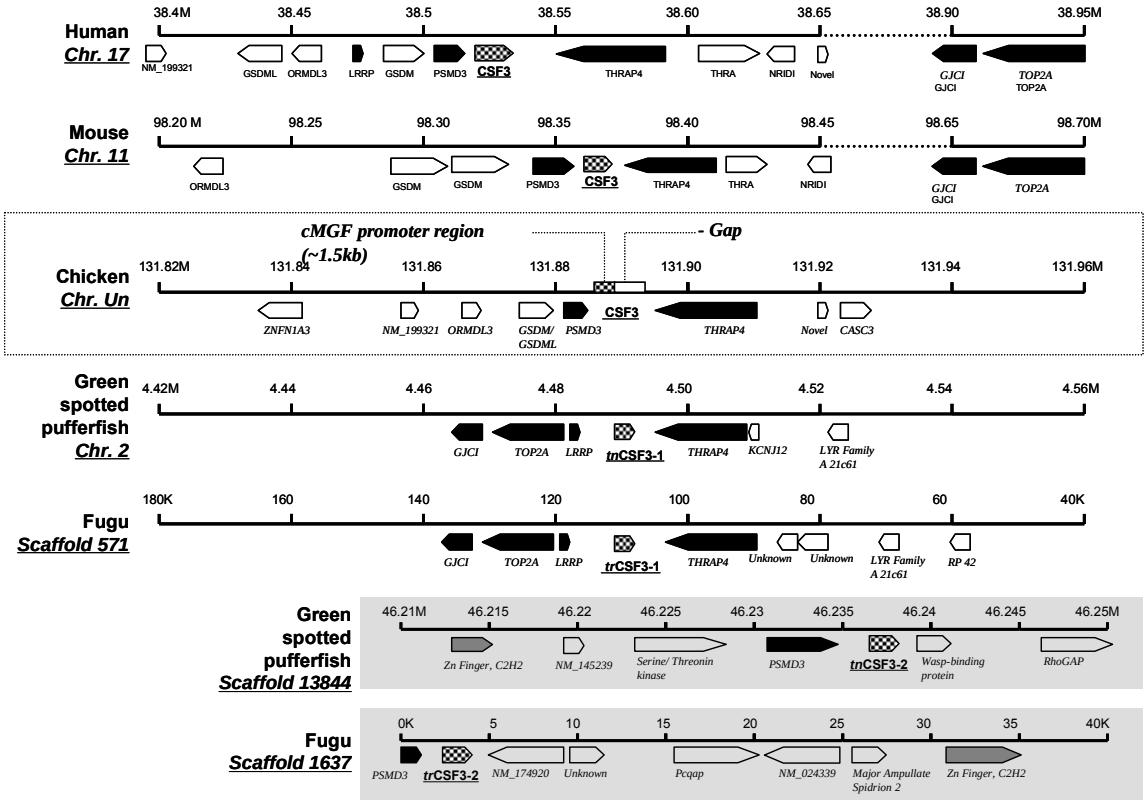

Figure 2. Syntenic putative CSF3 locus of the human (Chromosome 17), mouse (Chromosome 11), chicken (Gg Chr Un), green spotted pufferfish (Chromosome 2 and SCAF13844) and fugu (Scaffolds 571 and 1637). The putative location of the CSF3 gene (CSF3 in bold) is shown to be flanked by conserved (black and gray block arrows) and non-conserved (unfilled block arrows) genes. The putative chicken CSF3 locus is also included and shows the cMGF (M85034) promoter site (checkered rectangular box) followed by a gap (unfilled rectangular box). The duplicate genes (CSF3-2) are shaded with gray to differentiate from CSF3-1s. Figure is not drawn to scale.

but conserved position of the GJCI and TOP2A genes of the said loci. This indicated that fish CSF3-1s were the 'original' or ancestral CSF3 orthologues and the CSF3-2s were the duplicates.

Phylogenetic analysis likewise revealed some interesting information (Fig. 3). First, all the CSF3s were found to form a single evolutionary clade (that includes cMGF) outside

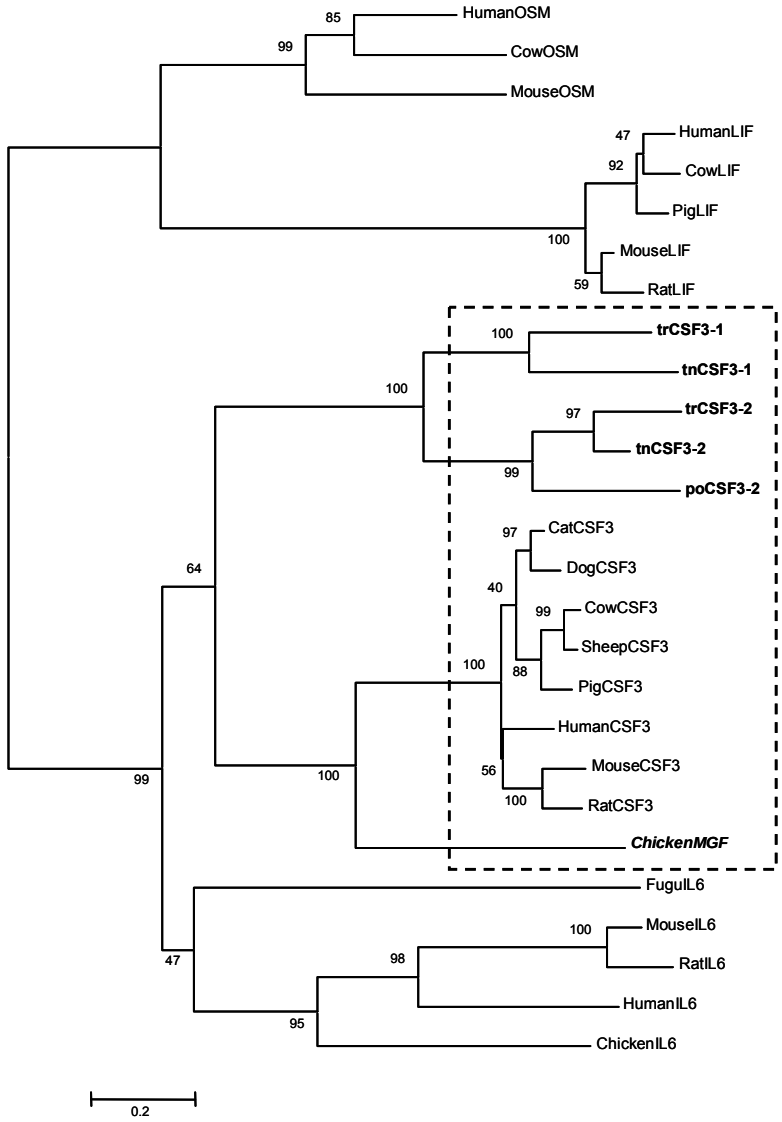

Figure 3. Neighbor-joining tree of the Pfam IL-6/CSF3/MGF protein family. Additional accession numbers: Human IL-6 (*NP000591*); Pig IL-6 (*NM_214399*); Mouse IL-6 (*NP112445*); Rat IL-6 (*NM_012589*); Human OSM (*M27286*); Cow OSM (*S78434*); Mouse OSM (*D31942*); Human LIF (*NM_002309*); Cow LIF (*NM_173931*); Dog LIF (*AF512028*); Mouse LIF (*NM_008501*) and Rat LIF (*NM_022196*).

of the sequence related IL6, OSM and LIF genes, suggesting that the CSF3s have a common ancestor and hence are indeed orthologous. Inclusion of cMGF in the CSF3 clade provides additional evidence that it is the chicken CSF3 orthologue. Second, the phylogenetic trees are also in accord with our claim that the fish CSF3s genes exist as

paralogs. Third, the divergence of the other members of the SCOP IL6/CSF3/MGF protein family is well placed before the mammalian-fish divergence, and IL6 is more related to CSF3 than to OSM and LIF

The teleost CSF3 genes, except *tn*CSF3-1 were found to be functional, with different expression patterns in tissues (Fig. 4). *po*CSF3-2 was expressed constitutively in Japanese flounder tissues known to have immuno-hematopoietic-related functions: gills, kidney and spleen. Interestingly it was also active in the brain and ovary, although at a

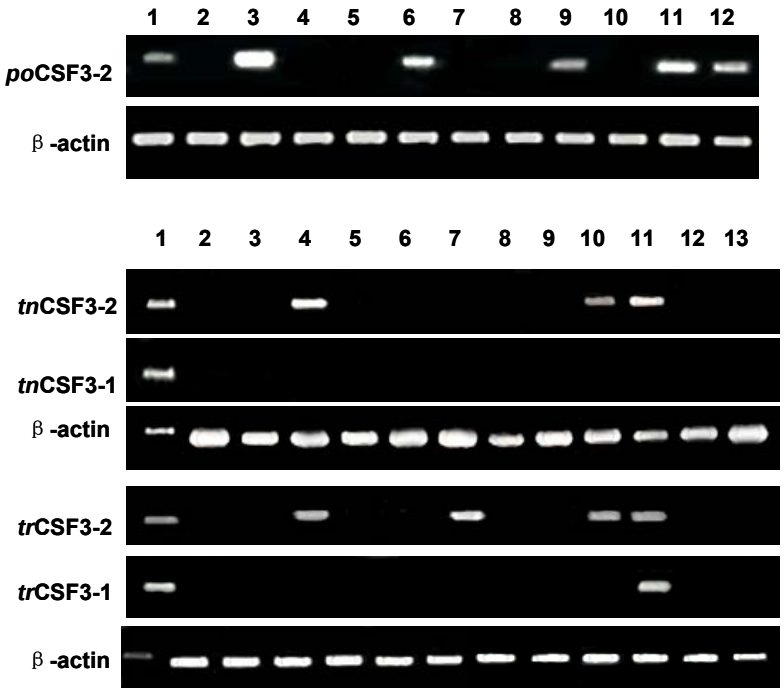

Figure 4. Constitutive expression of teleostean CSF3s in various tissues/ organs in Japanese flounder, fugu, and green spotted pufferfish. Lane M – 500 bp marker, Lanes 1 – brain, 2 – eyes, 3 - gills, 4 – heart, 5 - intestine, 6 – kidney, 7 – liver, 8 – muscle, 9 – ovary, 10 - skin, 11- spleen, 12 – stomach. β-actin was used as a control. Expression of fugu and pufferfish CSF3s in tissues. 1- 500 bp marker; 2 – Brain; 3 – Eye; 4- Gills; 5- Heart; 6- Intestine; 7- Kidney; 8- Liver; 9-Muscle; 10- Ovary; 11- Skin; 12- Spleen; 13- Stomach.

lesser degree. *tr*CSF3-1 were expressed in gills, kidney, ovary and skin while *tr*CSF3-2 in skin only. On the other hand, only one of the green spotted pufferfish CSF3 genes (*tn*CSF3-2) showed expression in gills, skin and ovary.

Expression of *po*CSF3-2 in kidney was evidently up-regulated at initial induction by LPS and ConA/PMA and then decreased gradually thereafter. In contrast, PBLs exhibited an increasing expression of CSF3 upon induction with Con A/PMA and a decreased activity at 6 hrs post induction with LPS (Fig. 5). To check expression of *po*CSF3-2 in response to poly I:C, we induced primary cultures of brain, kidney, PBLs and spleen of

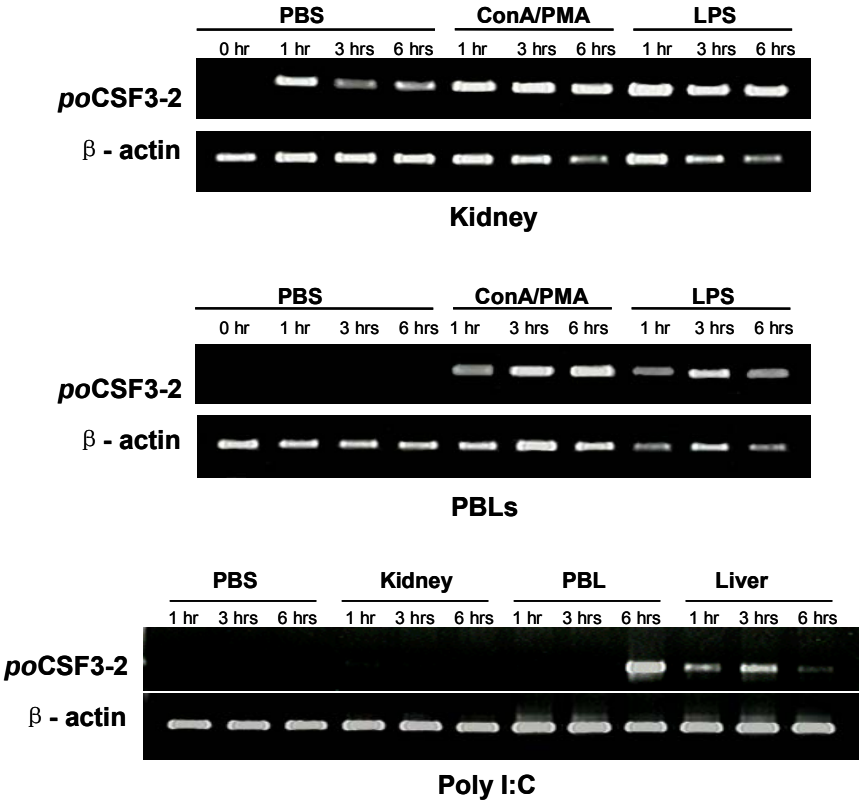

Figure 5. Expression of Japanese flounder poCSF3 gene in response to immunostimulants. (a) CSF3 gene expression in Japanese flounder kidney *in vitro* at 1, 3 and 6 hrs post-stimulation with LPS, ConA, PMA and combination of ConA/ PMA. (b) CSF3 gene expression in Japanese flounder peripheral blood leukocytes (PBLs) *in vitro* at 1, 3 and 6 hrs post-stimulation of LPS and a combination of ConA/PMA. Phosphate-buffered saline (PBS) was used as negative control. CSF3 expression in Japanese flounder brain (1-3), kidney (4-6), PBLs (7-9), and spleen (10-12) *in vitro* following poly I:C treatment at 1, 3 and 6 hrs incubation period.

Japanese flounder. *po*CSF3-2 expression was not detectable in the brain, only faintly observed in kidney at 1 and 3 hrs, highly expressed in PBLs at the 6th hr incubation and was inducible in spleen at 1 hr post-treatment.

3.2. Japanese flounder IL-11b

The complete Japanese flounder interleukin 11 type b (*po*IL11b) cDNA was composed of 1,536 bp encoding for a putative protein of 201 amino acid residues (Fig. 6). The nucleotide sequence showed 4 mRNA destabilizing AUUUA motifs and a typical poly-adenylation signal "AATAAA". The polypeptide on the other hand had a predicted signal sequence of 23 aa. Cleavage of this peptide results to a mature *po*IL11b protein containing 178 aa with a predicted molecular mass of about 20 kDa and predicted isoelectric point (pI) of 6.94. It was leucine (L) and serine (S) rich at 16% and 15%, respectively, had 4 C residues and 4 predicted *N*-linked glycosylation sites. Using the

```
CCACGCGTCCGTGTACACACACTGGAGTCAGAGGAGGTCGGTTCACTGCAGCAGGAGAAG   60
AAGAAAG***ATG***AAATTGCTTCATGACTCCATCCCATGTCTTTTCCACCTGCTGCTATTGGC  120
       M  K  L  L  H  D  S  I  P  C  L  F  H  L  L  L  L  A
TGAGCTGTTTGTCCCGTCATCGTCTCGTCCCGTCCACACCTCCTCCCTCTGTAGGATGTT  180
 E  L  F  V  P  S  S  S  R  P  V  H  T  S  S  L  C  R  M  F
TGGATCGATGATCCATCAGGTGGACAAGCTGACGGACATCTCCAAAAACCTCCATGAGCT  240
 G  S  M  I  H  Q  V  D  K  L  T  D  I  S  K  N  L  H  E  L
GTCGGACAACAACGAGCTCCTGAACTCTGCGGATAACAAACTTCCTGATCTTCCTCACAT  300
 S  D  N  N  E  L  L  N  S  A  D  N  K  L  P  D  L  P  H  M
GCAACACTCTGCGGCACATTTTTTTAATTCACTGAAGATGAACGAGTCCCTCTCTGAGCT  360
 Q  H  S  A  A  H  F  F  N  S  L  K  M  N  E  S  L  S  E  L
CTACCTGCTCGCTCAGGCCTTCAGGCTGCACGTCGACTGGCTGAAGACGGAAAAAGACAA  420
 Y  L  L  A  Q  A  F  R  L  H  V  D  W  L  K  T  E  K  D  N
CTTCAGTTTACCCAGTCAGTCAGCAGAGGACGCCAGCACTCATCTGCTGCAGCTGTCCAA  480
 F  S  L  P  S  Q  S  A  E  D  A  S  T  H  L  L  Q  L  S  N
CCTGCTCAACATGTCACTGCACCAGATGAGTGCAGAGACGCCTCAGCCGCCGGCTCCCTC  540
 L  L  N  M  S  L  H  Q  M  S  A  E  T  P  Q  P  P  A  P  S
CCTCCCTGTCGTCTCCTCGGCCTTCGACCTTCTCCAGTTCTCCATCGAGATCTCTGAACG  600
 L  P  V  V  S  S  A  F  D  L  L  Q  F  S  I  E  I  S  E  R
GCTAAAAGTCTTCTGTAATTGGTCAAAAAGAGTTCTACGATCTCTAAAACTCCCGCGCTG  660
 L  K  V  F  C  N  W  S  K  R  V  L  R  S  L  K  L  P  R  C
CCGCAGACAG***TGA***GAAGCAGCGGCTCATGTCTTCCTAACCACTGTTAACGTAAAAGATTT  720
 R  R  Q  *
TCATGCAGCAGCGAATCAGCAGCAAAACTCTGGAGGCAGGAGAGTCACTTTGGTTCAACA  780
ACAATAGATATATACTTTGCACTGTGCTCAGAGGATGTGAACCCTCGACCACACACACGT  840
TCACTTATCGGTTTTATTTCAACGCCTTGTTGATGAATTGGTACAAAATCTGGACGTTGA  900
CAGTGAAGAATTTTCATGACTTCGGTGATTTGCTTCCTCAGACGGCATCAGGTTTACATG  960
TGGTTTTTTGAAATAAGTCTGAGTTTGGTTCGGATGTGTTTTGTTTGGACAAGTTAGGAC 1020
AAAATGGTGAATGTTGAAGTAACTATTGGTGGTGGGAACATTATGGGAAGTTTACCCCCA 1080
GATGATCAACTTTTAAAGGTCGACAAAAATATGGTCTCAAAAAACAGTCGCAAATAATAG 1140
AGACTAAAGTTCGTGTTGATCAGGAAATGATTGTGTGAGGTGCAGCGCTCAGTGGCACGG 1200
TTACGTTTACGCCCTTTAAGACTGTTTACATGATTCAAAGTACGAGAAAAAATATGAATG 1260
AATGCTGTTATTAATATTTTTCTACTGACTGTTTTAT***ATTTA***AGAGGCT***ATTTA***TGATAT 1320
TTGTTACATAAAGAACTTGACAGTGTTTAGTGTGAGATGCAGGCATCTCATTGTTTTCTA 1380
TGAAAT***ATTTA***ATATTAATATTAATAATAATAATAACGTATTGTCATGATGTAGACGGTT 1440
ACACTTCT***ATTTA***TACTTTTTATACCTTTTTGTTTTTCTACCATGTTTGAAGTCATT***AAT*** 1500
***AAA***AATTTACATTAAAACATAAAAAAAAAAAAAAAA                      1536
```

Figure 6. Complete cDNA sequence of Japanese flounder IL 11 type b (*po*IL11b). Start and stop codons are in bold, italized; predicted signal peptide is underlined, potential *N*-linked glycosylation sites are boxed; the mRNA destabilizing motif ATTTA are in bold, underlined; and the poly(A) signal AATAAA is in bold.

ProDom and 123D+ servers, *po*IL11b protein has been identified as homologous to the mammalian IL11 motif and belongs to the Structural Classification of Proteins family of long-chain cytokines (A.26.1.1), respectively. Alignment of fish IL11 orthologues and paralogues revealed some interesting results (Fig. 7). The *po*IL11b leader peptide of 23 aa was 3-less than the other fish IL11a and 1-less than mammalian IL11s. Three C residues of *po*IL11b were shown to be part of the mature peptide. One C residue (C^{183}) was well conserved in fish IL11s but the other 2 C residues (C^{12} and C^{198}) were only conserved among IL11bs [*po*IL11b, tiger pufferfish (*tr*IL11b) and green spotted pufferfish (*tn*IL11b)] and not with IL11as (*tr*IL11a, *tn*IL11a, *cc*IL11a, *om*IL11a, *dr*IL11a.1 and *dr*IL11a.2). It is important to note that there are 2 zebrafish *dr*IL11s identified so far that are homologous to type a. The putative *po*IL11b helices (A to D) were mainly composed of charged residues aspartic acid (D), glutamic acid (E), lysine (L) and the polar histidine (H). Some residues that have been reported in mammals to be important for receptor binding were conserved in *po*IL11b including L^{28} and L^{34} in Helix A, L^{173} at the start of Helix B, L^{115} in Helix C, and tryptophan (W^{165}), arginine (R^{168}), L^{171} and L^{173} in Helix D. Another important residue which was conservatively replaced to phenylalanine (F) is the hydrophobic L^{170}. The comparative identities among fish and mammalian IL11 orthologues as well as isoelectric points (pI) were also interesting (Fig. 7). Between *po*IL11b and the other fish IL11b, the identities were significantly conserved at more than 50%. *po*IL11b has limited identity compared to IL11a orthologues (24% to 29%), and no significant identity with mammalian IL11s. Teleost fish IL11bs were also neutral compared to the IL11as, which are basic including the mammalian IL11s. With the complete *po*IL11b amino acid sequence and the limited identities especially between Japanese flounder *po*IL11b and 2 zebrafish IL11as, we ran a phylogenetic analysis with fugu IL6 as an outgroup. Our results confirmed the clustering between IL11as and IL11bs and hence the orthology of *po*IL11b. Moreover, we also show a, lineage-specific, secondary duplication for zebrafish IL11a (Fig. 8).

```
                                                α-helix A
                                                     *       *
FlounderIL11b     MKLLHDSIPCLFHLLLLAELFVPSSSRPVHTSSLC-----RMFGSMIHQVDKLTDISKNLHELSDNNELLNS-ADNKLPD
FuguIL11b         .DVIE..A...L..F.....I.H.A...ASSPAP.-----.T.R.IF....L.MGL.RK..D...EDV.MFESME.R.DT
PufferIL11b       ...ID..A.N.LC.......I.H.TC..AGGAA..-----TNLK..L....R.IGL.SK..G...EEV.IIARME.S.DS
ZebrafishIL11a.2  ...SP..TFP.II.MACV...DFIRA..ANLPQGK-----KHLSTLYQDMRM.LKLT-----SQQM.ANELTDFEHS.SS
FuguIL11a         ....L..SSS.LFS....Q.P.FV.AS..PHRRP------SDMDRLSN.TKH.MKLT---Q..LREHSFDSDVEPHRFTS
PufferIL11a       ....L..SSS.LFS....Q.PLLT.TS..PHRRP------SDMDRLSN.TKH.IKLT---...LKDHSFDSDVEPH.F.S
TroutIL11a        ....V..SSS.LLS....QIPLFT.AV.APYRRPNV---VHELDRLAN.TKN.RQ.T---AD.LKEHAFETDPEQHRFKS
CarpIL11a         ....G..SSS.LLS....Q.HLLA.AF.A.PRRIQ-----TD.DKLSN.TRH.LKLT---QD.LK.PVFATEIDHQRFKS
ZebrafishIL11a.1  ....G..SSS.LLS....Q.HLLA.AF...HRRNQ-----ID.DKLSN.TKL.LTLT---RN.LKDRVFSTEINHHRFKS
MouseIL11         ---MNCVCRLVLVV.S.WPDR.VAPGP.AGSPRVSSD-PRADLD.AVLLTRS.LADTRQ.AAQMRDKFPADG--.HS.DS
HumanIL11         ---MNCVCRLVLVV.S.WPDTAVAPGP.PGPPRVSPD-PRAELD.TVLLTRS.LADTRQ.AAQLRDKFPADG--.HN.DS

                                  α-helix B                                  α-helix C
                     *       *  *                                           *
FlounderIL11b     LPHMQHSAAHFFNSLKMNESLSELYLLAQAFRLHVDWLKTEKDNFSLPSQ-SAEDASTHLLQLSNLLNMSLHQMSAETPQ
FuguIL11b         ...LP..-.EY.R.F.V.....Q.S.HT.S..Q.I....LARE.V....R-A...S.....K......A.....NE.V..
PufferIL11b       ...L...-.EY.R..QV...F.Q...QTES..Q......RAQ..C.....-A.RSS.A......K..DA..Q..DQAV..
ZebrafishIL11a.2  ..SLNY.-VKDLH..EVSST.AQ..SGLKS.KF.L..VQRNS.E----LGNDYSKTKKIVHLIQAIIQKV.QELGQTA.E
FuguIL11a         ..E.SNRS..SL.N.ELKPT..Q.HADLKLYEH.FE..NRVSKKHHH.ALPKLVEMIKEMKS.IT..HCQMLRVE.PRLT
PufferIL11a       ..E.SNRS.NDL.N.ELKPT..Q.HAELKLYEH.FE..NRVSKKHHH.ALPKLVEMIKE.KS.IS..HHQMLRVE.PRLN
TroutIL11a        ..L.NNR-.SDI...E.RPT..Q.HADLKS.EH.FA..SRASRKHHH.ALPKLGQMMSLIKS.TSM.EHQMMRVD.QRLS
CarpIL11a         ..AISSR-VSDLTT.EFKPT..Q..ADLKS.EH.FE..NRTTRKQQHS.VPKLT.MIS.IKS.I.S.QRQMTRAE.PRIP
ZebrafishIL11a.1  ..AISSR-.SD.AT.EVKPT..Q.HANLKS.QH.FE..NNITHKQQHS-IPKLT.MVS.IGG.V.S.QRQMNHIG.PRLP
MouseIL11         ..TLAM.-.GTLG..QLPGV.TR.RVDLMSYLR..Q..RRAGGPSLKTLEPELGALQAR.ER.LRR.QLLMSRLALPQAA
HumanIL11         ..TLAM.-.GALGA.QLPGV.TR.RADLLSYLR..Q..RRAGGSSLKTLEPELGTLQAR.DR.LRR.QLLMSRLALPQ.P

                                     α-helix D                                     (%)   pI
                              *                *  *    *
FlounderIL11b     PPAPSLP--VVSSAFDLLQFSIEISERLKVFCNWSKRVLRSLK-LPRCRRQ-------------------    -    6.94
FuguIL11b         L.PL...--IA.TS..V....V...D...I..H.......Y.QR.N..PKH-------------------    59   6.69
PufferIL11b       A.L..F.--....S.EV....V...DQ.T...D.T.....AVQRVS..PTPTLRLRG-------------    56   6.72
ZebrafishIL11a.2  IVH.T..P--LETFWQ.Y.TNA..HKK.LI..DYYT.A.G...RKHPDTPS-------------------    27   8.65
FuguIL11a         .AT....P-HLPYQ..V..S.H.LLQHF.L..D.AY.AFL...PKVN-AAVQ------------------    24   9.43
PufferIL11a       LTT....P-QLPYQ..V..S.H.LLQHF.L..D.AY.AFI...PKV--SAVQ------------------    26   9.38
TroutIL11a        ..S..M.P-PPP.Q..V..S.Q.LLLQFRL..D.AQ..FSV.STKSKMSAVQ------------------    25  10.23
CarpIL11a         V.S....P-NPAFHWEVV.S.Q.LLQQFRL..D.AS..FLT..SKLP--A--------------------    25  10.40
ZebrafishIL11a.1  V.S....P-IPAFHWEMV.T.Q.LL.QFSL..D.AA...GRTRS.LTS.EAPVVGSTGTSPSGPIRIVGK    29  10.82
MouseIL11         .DQ.VI.LGPPA..WGSIRAAHA.LGG.HLTLD.AV.G.LL..TRL------------------------    NI  11.38
HumanIL11         .DP.AP.LAPP...WGGIRAAHA.LGG.HLTLD.AV.G.LL..TRL------------------------    NI  11.09
```

Figure 7. Alignment of Interleukin 11 orthologues and paralogues. Signal peptides are boxed while charged amino acids (aspartic acid, D; glutamic acid, E; lysine, K; and arginine, R) and the polar histidine (H) are shaded in black. α-helices A to D are shaded gray. Conserved residues are represented by dots (…) while gaps (-) were introduced to maximize alignment. *NI* means no significant identity.

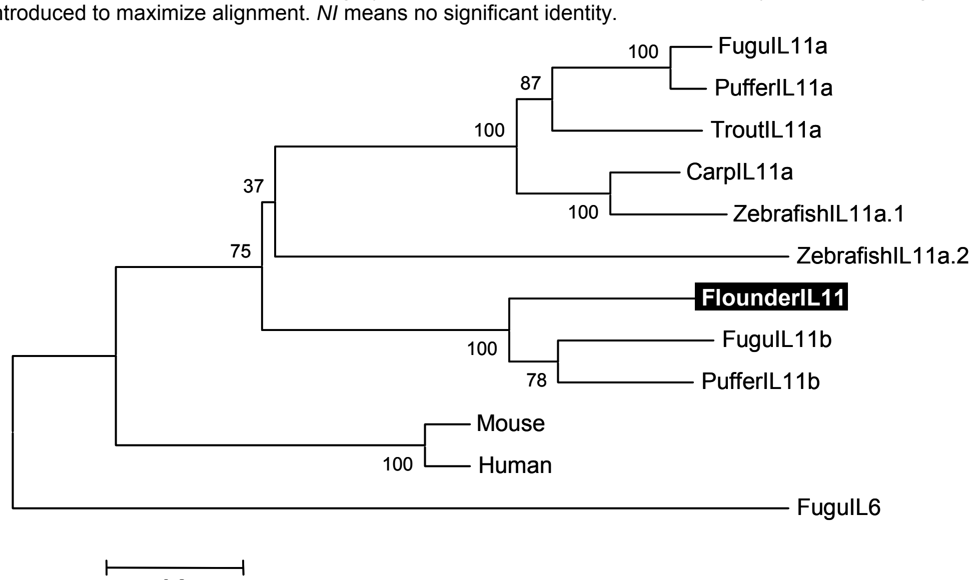

Figure 8. Neighbor joining tree of the Japanese flounder IL 11 type b and other IL11 orthologues and paralogues. The JTT Matrix (Jones-Taylor-Thornton) method was utilized. Accession numbers include: Japanese flounder IL11b – AB299205; Fugu IL11a-BN000713; Fugu IL11b- BN000714; Green spotted pufferfish IL11a- BN000715; Green spotted pufferfish- AY374548; Zebrafish IL11a-BN000717; Zebrafish IL11b-BN000718; Trout IL11a- AJ535687; Carp IL11a-AJ632159; Mouse IL11-P47873; Human IL11-P20809.

At 30 PCR cycles, Japanese flounder *po*IL11b was remarkably expressed in PBLs and very slightly in kidney and spleen but not in brain, eyes, gills, heart, intestine, liver, muscle, skin and stomach (Fig. 9)

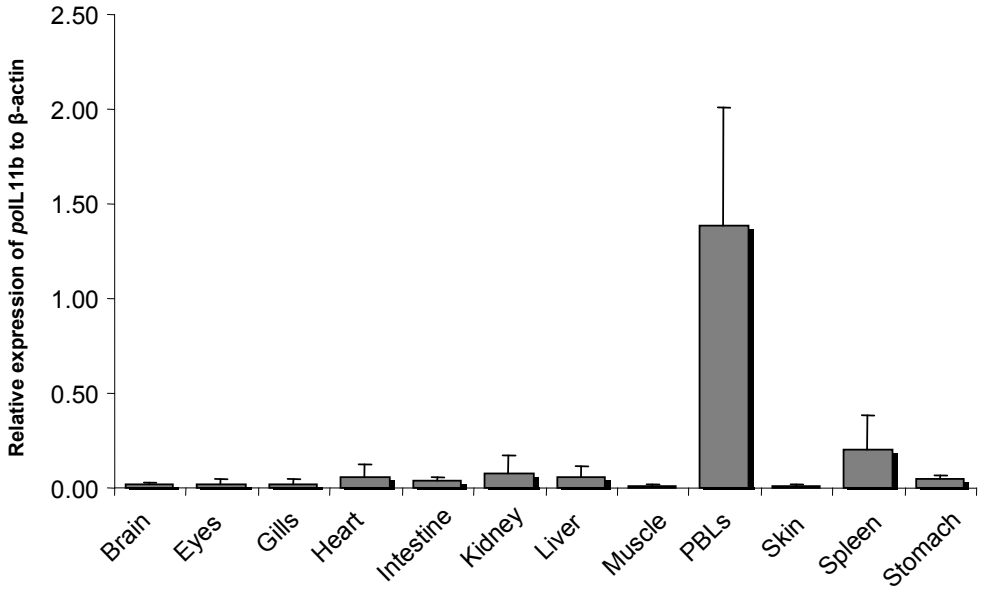

Figure 9. Constitutive expression of *po*IL11b gene in various tissues in Japanese flounder relative to β-actin expression as determined by RT-PCR.

We checked the expression of *po*IL11b vis-a-vis with the Japanese flounder granulocyte colony stimulating factor (*po*CSF3), Mx (*po*Mx)and β-actin genes in primary cultures of kidney, PBLs and spleen of adult fish following LPS and poly I:C treatment. Expectedly, being a pro-inflammatory molecule, *po*IL11b was induced by LPS although not comparable to *po*CSF3 in the 3 tissues studied. Surprisingly, however, it was highly induced by poly I:C in a time-series manner similar to *po*Mx expression (Fig. 10). Japanese flounder *po*IL11b was expressed, albeit very slightly, in the kidney of juvenile Japanese flounder infected with *E. tarda* starting at day 3, in contrast to *po*CSF3 where there was visible expression starting at day 1 and moderately expressed at day 3 and day 7. On the other hand, kidney of juvenile fish infected with HIRRV showed increasingly high amounts of *po*IL11b transcripts as compared to PBS-injected controls starting from day 1 until day 7. This result was confirmed by *po*Mx expression, where poly I:C is

known to stimulate it, and by the constant expression of β-actin. Unexpectedly, from this experiment, we also noticed that *po*IL11b is not constitutively expressed in the kidney of juvenile Japanese flounder (Fig. 11).

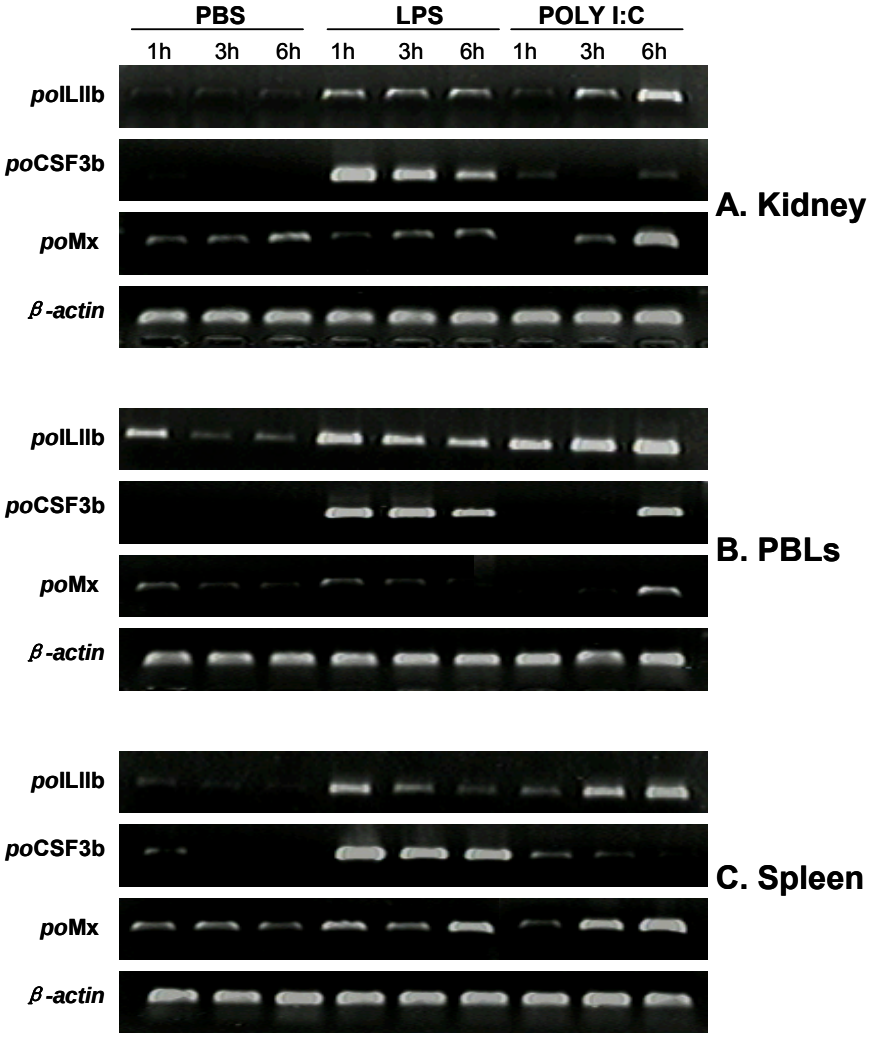

Figure 10. Expression of *po*IL11b, poCSF3, poMx and β-actin genes in primary cultures of A) kidney, B) peripheral blood leukocytes (PBLs) and C) spleen following PBS, LPS and PolyI:C treatment *in vitro* at 1 hour (1h), 3 hours (3h) and 6 hours (6h) sampling.

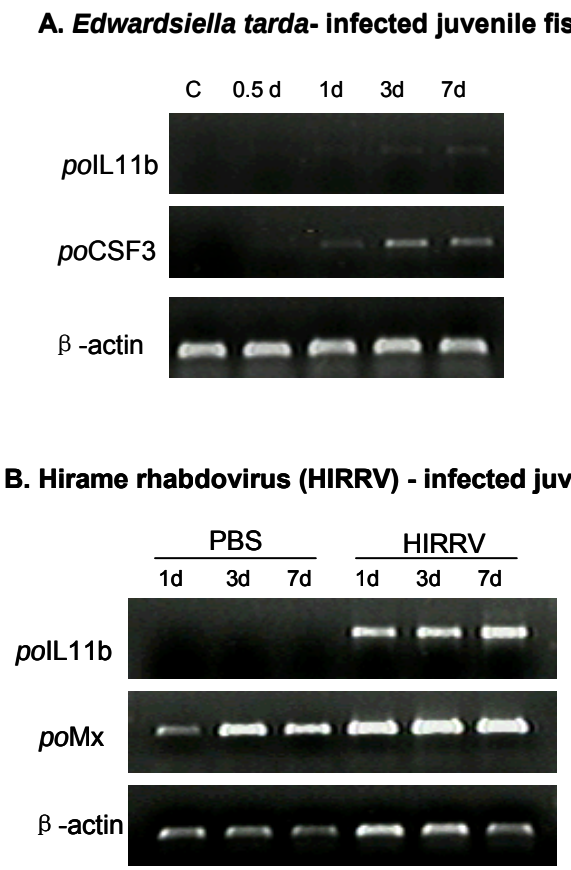

Figure 11. Expression of *po*IL11b gene in kidney in response to *Edwardsiella tarda* and Hirame Rhabdovirus (HIRRV) infection *in vivo*. Sampling was conducted at 0 day (control, C), half day (0.5d), 1 day (1d), 3 days (3d) and 7 days (7d) post-infection for *E. tarda.* For HIRRV experiment, sampling was done at 1 day (1d), 3 days (3d) and 7 days (7d) post-HIRRV infection and for PBS

3.3. Japanese flounder M17 homologue (MSH)

We identified a complete cDNA sequence of a cytokine (AB280428) having close homology (41%) with carp M17 from the screening of a Japanese flounder head kidney cDNA library (Fig.12). It consisted of 1,006 bp encoding 215 amino acid residues with two putative *N*-glycosylation sites (arginine, N^{81} and arginine, N^{138}). The first 28 amino acids, cleaved between alanine (A) and valine (V), were predicted to constitute a signal sequence by the software SignalP. The 3' UTR had 5 AUUUA mRNA destabilizing motifs and contains the consensus polyadenylation signal ATTAAA. Since the molecule has significant identity with carp M17, we named it as Japanese flounder M17 homologue (*po*MSH).

By searching available fish genomic databases using *po*MSH as query sequence and doing comparisons, we located and fully annotated the orthologue of MSH in tiger puffer fish (*tr*MSH)- CAF99247, green spotted pufferfish (*tn*MSH)-O62728 and stickleback (*ga*MSH)-*Ensembl* code: ENSGACT 00000022426, while we correctly annotated the orthologue of M17 in carp (*cc*M17), goldfish M17 (*ca*M17) and zebrafish (*dn*M17-NW_634687) (Fig. 2.A). Alignment of these six fish cytokine amino acid sequences showed 4 conserved cysteine (C) residues and that the carp, goldfish and zebrafish M17s possessed 2 additional conserved cysteine (C) residues (Fig. 13). This alignment also showed 4 conserved amino acid motifs (*boxed regions*), parallel to the conserved protein domains detected by the ProDom server, that could potentially pertains to the 4 α-helices.

The signal peptides of the M17s and MSHs were predicted to be 32 a.a. and 29 a.a., respectively using Hidden Markov and Neural network models. These signal peptides were composed of numerous methionine (M) residues that are potential start codons.

We did BLAST search and identity analysis for M17 and MSH and found that these genes did not show any counterpart in higher vertebrates. The closest identity the BLAST search produced was with chicken CNTF and mouse CT-2, hence M17 and MSH

```
GTCGACCCACGCGTCCGATTTGCCTGAGATACCGAGGAGAGAATGAATGGTTATGTAAAG     60
                                                M  N  G  Y  V  K
AGAATGAGTTTTCAACAGTTTATGGAACTGACAACAACGTTACTCTCTCTCCTGCTGGTT    120
R  M  S  F  Q  Q  F  M  E  L  T  T  T  L  L  S  L  L  L  V
ATGGCTGTTGATTCAACGAGGACTGTGGCAGTGAGCGGAAGCCAGCAGTGTGGGAATTCT    180
M  A  V  D  S  T  R  T  V  A  V  S  G  S  Q  Q  C  G  N  S
GTGCAGCAGACTTTAAAGCTCACCAGACTCTTAAAGAAGGAATCTGTTGACCTCATCGAA    240
V  Q  Q  T  L  K  L  T  R  L  L  K  K  E  S  V  D  L  I  E
ACATATAAGGCCTCTCAAGGAGAGATGTCAGAGGACCTCTGCAATTTGTCGGTCAACAAC    300
T  Y  K  A  S  Q  G  E  M  S  E  D  L  C  N  L  S  V  N  N
ATCCCTGACCCCAACATCTCTGGCCTGGAGCCGTCAGAGAGGATAGCGAGCATCTACACG    360
I  P  D  P  N  I  S  G  L  E  P  S  E  R  I  A  S  I  Y  T
CACCTCCAAGCCTTCCTCACACATTTCAAACGGGTGTACGAGCAGCAGACAGACTTGCAG    420
H  L  Q  A  F  L  T  H  F  K  R  V  Y  E  Q  Q  T  D  L  Q
TTACCATCGAACCCGCTGCTGGCTGAGCTCACCAATGTCAGCACCCGCAGCAGGAATCTG    480
L  P  S  N  P  L  L  A  E  L  T  N  V  S  T  R  S  R  N  L
GCATCTCTCATAAACAGCTTCTACCAGAGCCTCTTCCCAAACCTGCCCATGCCTGAGCCA    540
A  S  L  I  N  S  F  Y  Q  S  L  F  P  N  L  P  M  P  E  P
GCAGGGGGGCCCACGACGCTACCTCCGCCTCAGAACGTCTTCCAACAGAAGGTCTACGGC    600
A  G  G  P  T  T  L  P  P  P  Q  N  V  F  Q  Q  K  V  Y  G
TGTGCGGTCCTAAAGACCCATTTAGAGTTCCTGTCAAATGTCTACAGAGAACTGAGAACC    660
C  A  V  L  K  T  H  L  E  F  L  S  N  V  Y  R  E  L  R  T
CTAAAGAGCAAAGTCTGCAGGGGGATATAGACGAACGCACCCTTCTTCTGAGGATGACCT    720
L  K  S  K  V  C  R  G  I  *
AAGGCTGGCCTTAGACCGTCAGTTTCCAACAACACCTGTTCTCACTGGGTTTTGATAAAG    780
ACTCGCAAAGATGTTTGAAGGTGGCTGGTACATCAATGTAATGGAGCCCATTTATGGCAC    840
TTAATATTTATTTTCTTTTGTGTTCCAAATGAAGGGTGTGAATCCATCTAATTCATGTAT    900
ATTTAATTCATTTGATGTTATATAAGTATCTATTTTGCTGATGTAAACTTATTTACTGCA    960
TATGGAATTATTAAAAGAAAATTTAAAGCTGAAAAAAAAAAAAAAA                 1006
```

Figure 12. Full length of Japanese flounder MSH cDNA sequence. The putative signal peptide is *underlined* and *N*-linked glycosylation sites are *boxed*. In the 3'UTR the RNA instability motifs (ATTTAA) are in *bold* and the polyadenylation signal (ATTAAA) is in *bold underlined*.

```
Japanese flounder MSH  MNGYVKRMSFQQFMELTTTLLSLLLVMAVDSTRTVAVSGSQQ--CGNSVQQTLKLTRLLKKESVDLIETYKASQGEMSED
Stickleback MSH        ...H..SLR..P...PA.............S....ATRN..--.E..L...WRIA..VQ....E..K...T....NA.L
Tiger pufferfish MSH   ...H..SVHL.LX..AAAA..C.R.AV.....GG.MA..A.D--.A-L......I...VH...A...K..........F
G.s. pufferfish MSH    ...H.RS.Q..RQ.KAAAL..C.W......A..STTA..K.AGD.----SRA..I.EV.L...E...KI..S...Y...L
Carp M17               -----MVCLS.RSQAKFRMI.AI.ILI..ELVHPTVSCKNET--.SQLLRHS.R....MS.RTTE.L....T..-DFADL
Goldfish M17           -----MVCLS.RSQATFRMI.PV.ILI..ELVHPTVSCKNES--.SQLL.HS.R....TS.RTKE.L........DFADL
Zebrafish M17          -----MLCLS.RLQVKFRAYFAIIILI..QLVQPTMSCKNEN--.SQRLHRS...NKFTN.IT.K.LD.......DSTDL
                                                                  *

Japanese flounder MSH  LCNLSVNNIPDPNISGLEPSERIASIYTHLQAFLTHFKRVYEQQTDLQLPSNPLLAELTNVSTRSRNLASLINSFYQSLF
Stickleback MSH        ..KA.F.Y...........W..LL...K...D.FP..S.....M...S.ASR.....NRTNGGR....A..S.S.....
Tiger pufferfish MSH   F.KV.PSD..V.......Q.D.....S.Q.R..FP..R......S...P.TSA..SL.DV.RG.N.....VV..L....Y
G.s. pufferfish MSH    R.KMPQS.V.N...A.......M...S......FP..R..H...S...P.TS--S.RAQR..GQGHQ.P------VPAPL
Carp M17               I.DMQMD.V.VSTV..QTI.Q..L.V....KE..P.M.T.M...K..NP.T..VAEG.NRMI.HV.HI.VRV.CILEI.Q
Goldfish M17           I.EMQLD.V.VSTV..QTI.Q..L.V....KE..P.M.T.M......NP.T..VAEG.NRMI.HVTHI.VKV.CILEF.Q
Zebrafish M17          I.EMQMD.V.VST...QTE....L...S..K...P.L.T.M...R..DP.T..VTEGINSLI.HV.HM.VRV.CLL.I.Q
                                                                                                    *

Japanese flounder MSH  PNLPMPEPAGGPTTLPPPQNVFQQKVYGCAVLKTHLEFLSNVYRELRTLKSKV-CRGI--------
Stickleback MSH        ..............S.............Q....YR.LV...C...K.M....-..SRKPVN----
Tiger pufferfish MSH   ....G.G.K.A..KP..R..I.......V....YK......M..I.M...N.-.A.RMKRNVQLF
G.s. pufferfish MSH    .QPAGAPAR....K.....Y.....I...V..N.YKN.....KS..N...K---.P----------
Carp M17               ..I.I....ES..GI.HA..I....A...I..TRLQ.L..QAVQ.QKS..KGKM.RKSTKNGS--
Goldfish M17           ..I.I....ER..GI..A..I....A...I..TRLQ.L..QAVQ.QKS..KGKM.RKTTKDGS--
Zebrafish M17          ..I.I....ER..GI..A..I.......I..TRLQQL..QAVQ.QKS..-GKT.RRTKKNYS--
```

Figure 13. A) Multiple alignment of carp, goldfish and zebrafish M17, and Japanese flounder, tiger pufferfish, green spotted pufferfish and stickleback MSH. Signal sequences are *boxed-gray*, conserved cysteine residues are indicated are *boxed-black*, cysteine residues for M17 only are with asterisks (*) and conserved motifs are *boxed*. B) Identities among fish M17s, MSHs, and with mouse CT-2 and chicken CNTF (*filled-gray*).

appeared to be present only in fish and not in higher vertebrates. Furthermore, only the M17 group showed some level of homology (22% to 26%) to mouse CT-2 and chicken CNTF while the MSH group did not, except for *po*MSH, which has a 21% homology to mouse CT-2. Such pattern of homologies supported a separate orthology for M17 and MSH (Table 2).

Our analysis of the genomic structures in existing fish genome sequences revealed that zebrafish M17 (*dr*M17), tiger pufferfish (*tr*MSH) and stickleback (*ga*MSH) were composed of 3 exons and 2 introns, where the 3rd exon at the 3' end was considerably longer than the 1st and 2nd exons, similar to the published carp M17 (data not shown). The green spotted pufferfish (*tn*MSH), although it had a 4 exon-3 intron architecture, possessed essentially the same pattern with zebrafish, tiger pufferfish and stickleback because its 3rd exon, 3rd intron and 4th exon appeared to constitute the long 3rd exon in the latter species. This means that at the genomic organization level, M17 and MSH were similar.

Table 3: Identities of teleost fish M17, MSH and mouse CT-2 and CNTF

Tiger pufferfish *tr*MSH	G..S. Pufferfish *tn*MSH	Stickleback *ga*MSH	Zebrafish *dr*M17	Carp *cc*M17	Goldfish *ca*M17	Mouse CT-2	Chicken CNTF	Gene
64	52	70	43	41	43	21	---	Japanese flounder *po*MSH
	55	57	42	39	42	---	---	Tiger pufferfish *tr*MSH
		48	34	30	33	---	---	G..S. Pufferfish *tn*MSH
			37	39	38	---	---	Stickleback *ga*MSH
				74	74	22	26	Zebrafish *dr*M17
					91	23	25	Carp *cc*M17
						25	27	Goldfish *ca*M17

--- no significant identity

Genome analysis revealed that the *dr*M17 cluster of zebrafish included tescalcin, zinc finger matrin type 5, myosin heavy polypeptide 2, myosin heavy chain 4, frequenin homologue A, and the actin related 2/3 complex -subunit 5-like, while the *tr*MSH, *tn*MSH and *ga*MSH genes were determined to be conservatively flanked by myosin IC, sushi domain containing 2, serine/threonine-protein kinase, tescalcin, calcium-binding protein 7 and zinc finger and matrin type 5. Comparative synteny between the M17 and MSH clusters showed difference in their gene composition, again supporting an evolutionary separation between the 2 genes (Fig. 14).

A neighbor-joining (NJ) analysis based on the amino acid sequences of the fish M17s, MSHs and other related cytokines showed that fish M17s and MSHs clustering was supported by high bootstrap value at 99%. In addition, the M17/MSH grouped with CT1/

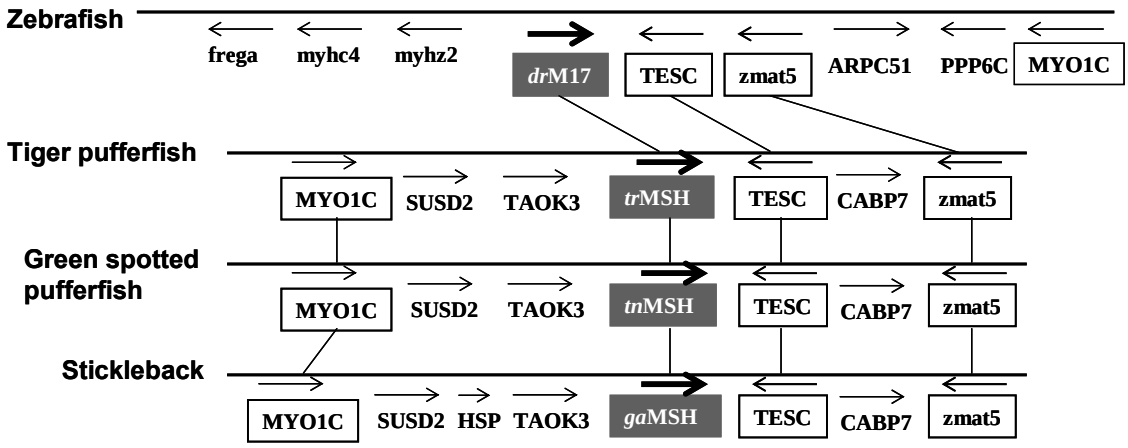

Figure 14. Synteny of the loci for zebrafish M17; tiger pufferfish, green spotted pufferfish and stickleback MSH. Flanking genes include: TAOK3 (serine/threonine-protein kinase), TESC (tescalcin), zmat5 (zinc finger, matrin type 5), SUSD2 (sushi domain containing 2), CABP7 (calcium-binding protein 7), MYO1C (myosin 1C), HSP (heat shock protein), myhz2 (myosin heavy polypeptide 2), myhc4 (myosin heavy chain 4), frega (frequenin homologue A), ARPC51 (actin related protein 2/3 complex, subunit 5-like), PPP6C (serine/threonine protein phosphatase 6, catalytic subunit).

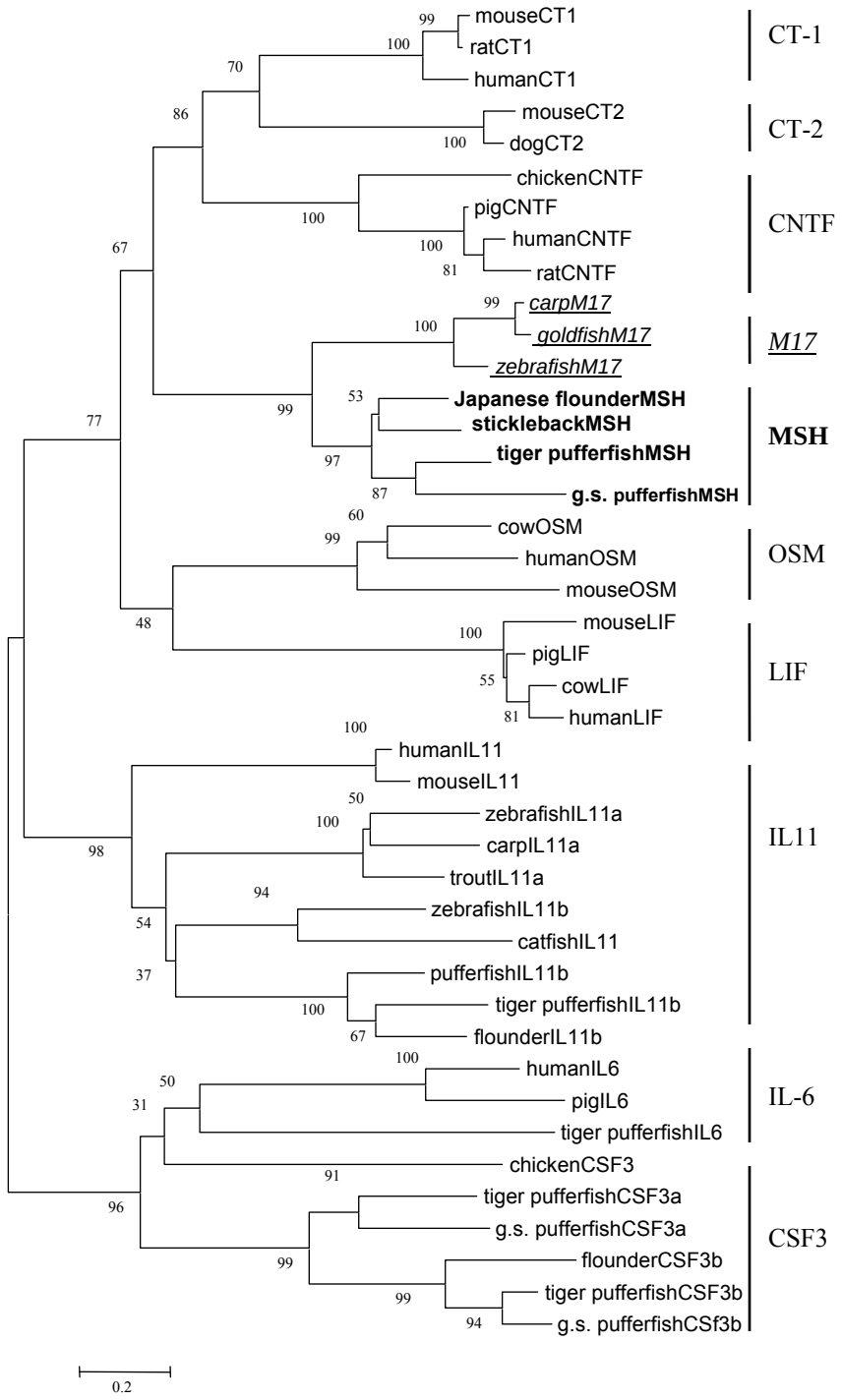

Figure 15. M17: carp (AAM52337), zebrafish (XP_684795), goldfish (DQ861993); MSH: Japanese flounder (AB280428), tiger pufferfish (CAF 99247); green spotted pufferfish (O62728); stickleback (Ensemble: ENSGACT00000022426); CT1: mouse (Q60753), human (NP_001321), rat (NP_058825); CT2: mouse (NP_942155), dog (XP_547035); CNTF: chicken (Q02011), human (P26441), pig (002732), rat (P20294); OSM: human (P13725), cow (NP_783644), mouse (P53347); LIF: human (P15018), mouse (P09056), pig (CAC14463), cow (Q27956); IL-11: human (P20809), mouse (P47873), zebrafish a (BN000717), zebrafish b (BN7000718), tiger pufferfish a (BN000713), tiger pufferfish b (BN000714), green spotted pufferfish a (BN000715), green spotted pufferfish (AY374548), catfish (CAJ57446), trout (AJ535687), carp (AJ632159) Japanese flounder (AB299205); CSF3: Japanese flounder b (AB200968), Tiger and green spotted pufferfish (Santos et al., 2006); and IL-6: human (P05231), pig (P26893), tiger pufferfish (NM_001032722). Branch numbers correspond to percentage of bootstrap values at 1,000 resampling.

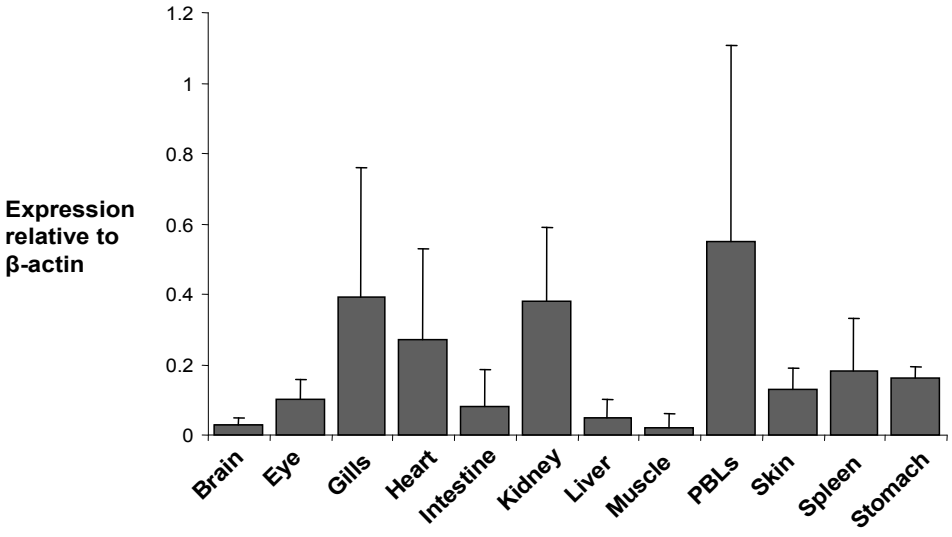

Figure 16. RT-PCR expression of Japanese flounder MSH gene in tissues of apparently healthy Japanese flounder. Values are relative expression of the MSH to β-actin.

CT2/CNTF, and this M17/MSH/CT1/CT2/CNTF cluster significantly separated with an OSM/LIF cluster at 77% bootstrap values. This suggests that M17/MSH is more closely related phylogenetically to CT1/CT2/CNTF than to OSM/LIF (Fig. 15). A tree constructed with the maximum parsimony showed similar pattern to the NJ tree (data not shown). The Japanese flounder *po*MSH was constitutively expressed in all the tissues examined although at varying levels using a 30 cycle PCR reaction. It was highly expressed in gills, heart, kidney and peripheral blood leukocytes (PBLs)and moderately expressed in spleen (Fig. 16).

Quantitative PCR revealed that Japanese flounder *po*MSH gene was significantly induced in kidney cells by polyI:C at 1 hr. post stimulationa. The gene was likewise up-regulated at very high levels by bacterial components, LPS and PG at 6 hrs after induction. Interestingly, the copy number of *po*MSH transcripts induced by PG is more than the LPS (Fig. 17).

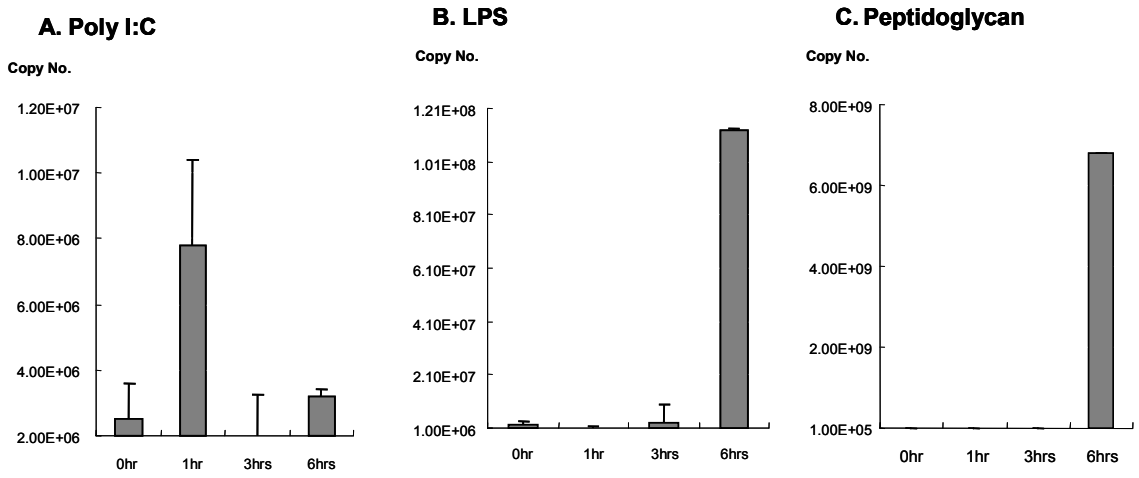

Figure 17. Quantitative real-time PCR analysis of Japanese flounder MSH in head kidney cells induced by (A) poly I: C (1 ug/ml), (B) LPS (1 ug/ml) and (C) peptidoglycan (1 ug/ml) sampled at 0 hr, 1 hr, 3 hrs and 6 hrs post-induction

4. Discussion

4.1. Granulocyte colony-stimulating factor (CSF3)

Here we established the first CSF3 orthologues from 3 different fish species (Japanese flounder, fugu and green spotted pufferfish) based on their conserved features, their synteny, phylogeny and expression. Such discovery of the fish CSF3 co-orthologues adds to the theory that fish has more genes likely caused by a whole-genome duplication event (Christoffels et al 2004; Jaillon et al 2004; Amores et al 1998) that could have allowed for teleost radiation and biodiversity (Ohno 1970; Postlethwait 2004). It also suggests to some extent, that myelopoiesis in fish and mammals, particularly the pathway being induced by CSF3 could be similar in both taxa because they both utilize the CSF3 molecule. Furthermore, our data shows that, CSF3-1s appear to be the descendants of the ancestral CSF3 gene and that the CSF3-2s are the duplicated copies. This would indicate that evolutionarily, CSF3-1s would exhibit the ancestral function as compared to CSF3-2s although actual functional analysis is needed to investigate this.

In spite of its rapidly evolving state, numerous CSF3 features were found to be conserved and could be important evolutionary functional elements. The consensus Pfam domains,

which covers more than 50% of the protein length for the tetraodontids and about 80% for Japanese flounder suggests that *po*CSF3-2, *tn*CSF3 -1 and -2, and *tr*CSF3-1 are likely to share the same secondary protein structure found in mammalian CSF3s and could hence share the same function. The presence of a concensus CK-1 decanucleotide sequence in lower vertebrates suggests that CSF3 may also be responsive to Tumor necrosis factor (TNF) - α, Interleukin (IL) - β (Shannon et al 1992) or LPS (Nishizawa and Nagata 1990). However, this needs confirmation since the equally important directly repeated NF-IL6 concensus elements that overlap downstream with CK-1 were not detected in lower vertebrates CSF3s and it is also unknown whether the CK-1 consensus sequence's proximity to the TATA box is relevant to its regulation of chicken and fish CSF3. The easily recognizable 3' UTR-linked mRNA AREs may play a part in mRNA regulation although it is unclear which type of AREs is involved in CSF3. UUAUUUAUU motif is said to be the shortest ARE that allows efficient mRNA deadenylation and decay, rather than the pentameric AUUUA sequences (Zubiaga et al 1995). However, our results show such UUAUUUAUU motif only in the fugu *tr*CSF3-2, the rest have pentamers. It is therefore possible that both these types are functionally relevant because it has been reported that there are AREs or mechanisms which could account for or complement mRNA decay (Yang et al 2003).

The presence of numerous simple sequence repeats (SSRs) or microsatellites interspersed in the untranslated regions (UTRs) and introns of the fish CSF3 genes may have contributed to this dynamic evolutionary state. As reviewed by Li et al (2004), various reports suggest that these SSRs are nonrandomly distributed in the genes and that they could interfere with correct gene transcription and translation thru frameshift mutations, slippage, SSR expansion and/or contractions which can inactivate or alter gene function and eventually allow for phenotypic changes. This limited identity could explain why the duplicate copy of *po*CSF3-2 was not detected by DNA hybridization and could very well be true with similar experiments in many genes in fish.

Aside from *po*CSF32 being partially identified from EST analysis of conA/PMA-stimulated Japanese flounder kidney cells (Arma et al 2004), its constitutive expression in

known immune-related organs of fish (kidney, gills and spleen) (Iwama and Nakanishi 1996) provides additional evidence that this cytokine is actively involved in at least the fish immune system. This is further confirmed by its inducibility by known mammalian CSF3 immunostimulants, endotoxin LPS (Sallerfors and Olofsson 1992; Hartung et al 1995 and Mathiak et al 2003), conA (Whitin 1987) and PMA (Oster et al 1989; Kothari 1995). In human, LPS started to induce monocytes to synthesize CSF3 3-6 hrs post stimulation (Sallerfors and Olofsson 1992). We therefore see a general similarity of fish and mammalian CSF3s activity in response to bacterial endotoxins, indicating that at the least, the gene's immune-related function is conserved. The different inducible expression of CSF3 in kidney and in PBLs in response to immunostimulants is quite interesting and warrants further study. At the transcriptome level, it appears that the fugu *tr*CSF3s and green spotted pufferfish *tn*CSF3-2 are likely to be functional while the *tn*CSF3-1 is not and might have become a pseudogene. *tn*CSF3-1 was previously reported to apparently lack the conserved Cys (C) residue, a missing probable receptor-binding-glutamic-acid residue (E_{19}), a rather long hydrophobic N-terminal region, an undetected conserved domain (Santos et al., 2006). If this is the case, the pufferfish paralogues probably followed a nonfunctionalization event, the classical theory of gene duplicate evolution, which predicts that one of the duplicate genes becomes fixated as a null allele incapable of transcription, translation or biological functions (Ohno, 1970). On the other hand, the fugu CSF3 paralogues were expressed although in different tissues. This suggests that the genes undergone subfunctionalization, also called the duplication-degeneration-complementation (DDC) pathway (Force et al., 1999). In the DDC pathway, the functions of both paralogues are complementary and have been preserved. The DDC pathway is not uncommon in fish. For example, evidence supporting the DDC theory has been observed in the *engrailed* genes of zebrafish. Another round of PCR (nested PCR) using some of the tissues for both fish yielded fragments of all four of the CSF3 orthologues (data not shown), indicating that all 4 mRNA transcripts are expressed and suggesting that pufferfish *tn*CSF3s is also undergoing positive selection. The functional evolution of the duplicate CSF3 genes in two species of the same fish family (fugu and green spotted pufferfish), which are thought to have diverged between 18 and 30 million years (Myr) ago (Hedges, 2002) needs to be clarified in future work.

*po*CSF3-2 production was observed in PBLs and spleen following poly I:C stimulation. In mammals, poly I:C has been reported to induce expression of CSF3 in endothelial and fibroblast cells (Fibbe et al., 1988b and 19899), and in uterine epithelial cells (Schaefer et al., 2005). In fish, poly I:C has been shown to stimulate immune-related genes such as viperin in mandarin fish (Sun and Nie, 2004), and the inducible isoform of nitric oxide synthase (iNOS) in small spotted cat shark (Reddick et al, 2006). Interleukin 6 (IL6) of fugu, which belongs to the same protein family as CSF3, and which is called Pfam IL6/CSF/MGF because of their conserved protein domains, was also significantly upregulated in the spleen when fish were injected with poly I:C (Bird et al., 2005). It is however unclear why CSF3 was not detected in brain and kidney, and in PBLs (after 1 hr and 3 hrs of incubation). In fugu, IL6 may have been increased in the kidney by poly I:C injection but the increase was not statistically significant. Nevertheless, this study provides indirect evidence that fish CSF3 is regulated by interferon or interferon-induced genes.

4.1. Teleost fish interleukin 11b (IL-11b)

Based on our analysis, the full Japanese flounder IL11 cDNA we report here is of type b (*po*IL11b) confirming previous partial third party annotation of this gene (TPA:CAJ57445) and results of phylogenetic analyses where the *po*IL11 EST fragment was included [Huising et al., 2005; Huising et al., 2006]. This suggests that there is also an existing IL11a orthologue in Japanese flounder that is yet to be identified as dictated by the Whole Genome Duplication (WGD) theory in teleosts (Christofells et al., 2004; Jaillon et al., 2004). Interestingly, the zebrafish possesses 2 *dr*IL11as as confirmed by identitiy, multiple alignment, isoelectric point and phylogenetic analysis. *dr*IL11a.1 is shown to be the paralogue of teleost IL11b and that *dr*IL11a.2 is the a paralogue of *dr*IL11a.1 at 31 % identitiy. These results indicate that *dr*IL11 gene has undergone an additional, secondary tandem duplication that is species-specific.

The general structure of *po*IL11b is conserved based on concensus domain predictions. It is similar with the other fish and mammalian IL11s because it is L residue rich and is made-up of 4 α-helices composed of charged amino acid residues that appear to be found at the hydrophilic surface of the protein molecule (Czupryn et al., 1995). Important receptor binding residues in *po*IL11b are also conserved suggesting that IL11 duplicates follow the same general receptor binding mechanism with that of mammalian IL11, which utilizes an IL11 receptor and gp130, and further shows that both paralogues could indeed be utilizing similar receptors that were recently predicted in pufferfish (Jaillon et al., 2004). A new type-1 cytokine receptor (Japanese flounder gp130 homologue, JfGPH) discovered recently could also act as a receptor for IL11s (Santos et al., 2007). Earlier studies reported that the helix A of mammalian IL11 particularly the P^{13}, E^{16}, L^{17}, R^{25}, L^{28}, T^{31}, R^{32}, L^{34} and R^{39} residues has been implicated in gp130 binding site (Site II) critical for bioactivity while Helix D particularly its R^{150}, H^{153}, D^{164}, W^{165} and R^{168} residues has been reported as a primary receptor binding site (Site I) (Du and Williams, 1997). In a more recent study, the R^{168}, L^{171} and L^{173} residues in the Helix D at the COOH-terminal have been found to be very important for binding of mouse IL11 to IL11 receptor, while the R^{111} and L^{115} residues were extremely crucial for binding to gp130. Furthermore, the W^{146} within the CD loop was determined to be critical for biological activity but not necessary for binding using A residue replacement experiment (Barton, 1999).

Despite the conserved IL11 structures, we were, however, able to spot some structural differences between fish IL11b and IL11a, which we think would have significant implication in their individual function. Firstly, there are 2 extra C residues conserved only among fish IL11b. These residues, particularly C^{183} and C^{198}, could potentially form a di-sulphide bond in this area and influence the stability of the peptide particularly the COOH-terminal of the protein. This could consequently affect the bioactivity of the molecule as this region is the primary receptor binding site (Site I). Secondly, IL11b orthologues are neutral compared to the basic nature of IL11a and even mammalian IL11s, which is a result of the presence of lesser charged amino acid residues in the helices and thus more neutral pI. IL11bs are therefore more cytokine-like in terms of pH

and the fish IL11as including mammalian IL11s are unusually basic. Thirdly, the *po*IL11b mRNA is composed of 4 destabilizing AUUUA motifs (Akashi et al., 1994) in its 3' UTR region and based on the presence of one poly (A) signal could only produce a single transcript. The DNA sequence of the trout IL11a on the other hand, has several AUUUA motifs and 4 poly (A) signals that potentially could produce different sized transcripts with varying stability depending on which transcript is produced (Wang et al., 2005). Assuming that the largest of the potential trout IL11a transcripts is transcribed (since this is the one cloned in the study), such IL11a is far more unstable than the *po*IL11b, which has only 4 ATTTA motifs. Taken together, these structural data suggests that fish IL11a and IL11b may very well exhibit different functional attributes.

The difference between IL11a and IL11b becomes clearer when expression data from both published reports and this study is compared, and that the difference appears to be complementary. First, studies show that carp *cc*IL11a and trout *om*IL11a is expressed significantly high in tissues except in PBLs (Wang et al., 1005; Huising et al., 2005). In contrast, we found that IL11b is highly expressed only in PBLs and very slightly in spleen and kidney. Second, *in vitro* studies showed that for LPS treatment, there is significant immediate expression of IL11b and is then dissipated afterwards while trout IL11a is persistently expressed at the duration of sampling. For poly I:C, a viral mimic (Fortier et al., 2004), the effect is more dramatic and increasing from 0h to 6h post-treatment, and approximates that of *po*Mx expression. This is in contrast to the expression of rainbow trout IL11a where it was observed to be slightly up-regulated by poly I:C at 3h and 7h sampling but then disappears at 24 hrs. Third, the results showed that *E. tarda* infection up-regulates *po*IL11b but not significantly, at least in the kidney of juvenile fish, unlike trout IL11a, which is up-regulated in the liver, head kidney and spleen of trout, 1 day after challenged with bacterial *Aeromonas salmonicida* MT423. HIRRV infection, on the other hand, stimulated high expression of *po*IL11b at 1 to 7 days sampling, again mirroring the expression of the antiviral Mx.

The fish IL11 paralogues appears to have complementing function, a characteristic that is very similar to fish M17s and MSHs, also members of the IL6-cytokine subfamily

(Hwang et al., 2007), that follows the Duplication-Degeneration-Complementation (DDC) model (Force et al., 1999). There is evidence to support, at least at the transcriptional level, of the partitioning of ancestral functions of these molecules. This model was initially supported by the analysis of the *engrailed* gene in zebrafish. Since then, a number of duplicate genes have been reported to follow such model including among others the *protocadherin 15* and αB-crystallin genes in zebrafish (Seiler et al., 2004; Smith et al., 2006) and the proopiomelanocortin genes in green spotted pufferfish (Souza et al., 2005).

Focusing on its immune function, *po*IL11b was indeed shown to be involved in both bacterial and antiviral responses *in vitro* and *in vivo*. Bacterial (LPS and *E. tarda*) and viral (polyI:C and HIRRV) mimics/agents have been shown to regulate numerous immune-related genes, including cytokines, during infection (Matsuyama et al., 2007; Yasuike et al., 2007). *po*IL11b is yet another cytokine molecule that responds to these agents and as such underscores its important role in host-pathogen interactions. This result adds to the growing information about fish IL11s. For example, trout *om*IL11a has been reported to be induced by a plasmid construct containing the glycoprotein gene of VHSV and a CMV promoter although the induction is significant only at day 3 in spleen and curiously only at day 10 in kidney (Jimenez et al., 2006). The non-expression of *po*IL11b, in juvenile Japanese flounder indicates that during development, it is functional only at the adult stage. A similar pattern has been observed with other genes related to hematopiesis where they are expressed at specific stages in zebrafish development (Davidson, 2004).

The viral challenge experiment and poly I:C treatment in trout, suggests that IL11a is not very much involved in antiviral responses unlike the *po*IL11b. It is therefore likely that IL11b has more antiviral function as compared to antibacterial responses and such broad involvement in pathogen responses makes it a good vaccine adjuvant candidate as compared to its counterpart IL11as in fish.

4.1. Teleost fish interleukin M17 homologue (MSH)

M17 has already been reported in carp, goldfish, zebrafish, green spotted pufferfish and tiger pufferfish (Huising et al., 2006). In this study, we isolated a cDNA molecule in Japanese flounder that is homologous to M17. However, we subsequently characterized it as a novel gene unique from M17 named as M17 homologue (*po*MSH) using comparative structural and expression analysis. With this and using bioinformatics, we classified the reported fish M17s to MSHs in tiger pufferfish, green spotted pufferfish and stickleback and M17s in zebrafish, carp and goldfish.

MSH was found to be unique from M17. Structurally, MSH has 4 cysteine residues while M17 has 6 suggesting a possible difference in their protein folding since cysteine (C) residues are know to form di-sulfide bridges. MSH does not possess significant identity with the chicken CNTF (except for *po*MSH with mouse CT-2) while carp, zebrafish and goldfish M17 have and the MSH loci cluster is not well conserved with the M17 loci. Phylogenetically, MSH and M17 are significantly separated at 99% bootsrap values. Constitutive expression also showed a striking difference between MSH and M17. *po*MSH is clearly expressed in all the tissues examined except in the brain, while M17 as shown previously in carp and goldfish, was detected in very high quantities in the brain, and to a lesser extent in kidney and blood leukocytes. Such differential expression suggests that MSH performs other and/or additional functions than M17. In addition, these data suggests that MSH is a gene closely related but separate from M17. Such gene evolution is not uncommon within the IL6 subfamily. For example OSM and LIF, considered to be genes with different functions, have high amino acid similarity and are thus thought to be a product of gene duplication (Huising et al., 2006). M17 itself has been reported to be of 2 types both located in chromosome 5 differing only by 2 amino acids (Hanington et al., 2007). Because of the close similarity of the 2 genes and of the widely believed genome duplication in teleost, we actually tried to investigate whether MSH is the duplicate copy of M17. Using *tr*MSH and *dr*M17, we did a "tBLASTn" search of the fugu and zebrafish genomic databases, respectively but we could not find a

"homologue" with significant E-value, identity and alignment nor did we find a cluster similar to the *tr*MSH and *dr*M17 clusters.

Since it is known that zebrafish and carp (Cypriniformes) are more primitive than the Japanese flounder (Pleuronectiformes), tiger pufferfish and green spotted pufferfish (Tetraodontiformes), and stickleback (Gasterosteiformes) (Miya et al., 2003), it follows that M17 is more primitive than MSH. Thus, it is tempting to speculate that MSH may have arisen from M17 by gene duplication similar to OSM and LIF. MSH could also be an evolutionary selected form of M17 since we could not find the duplicate copy of MSH in the available genomic databases. More information on these genes could clarify these issues.

The characteristic of fish M17 reported so far is confusing at best. It has been previously shown in carp to be structurally more related to OSM/LIF than CNTF because of the presence of signal peptides, position of the cysteins and similarity in exon/intron configuration. But it was at the same time said to be more similar to CNTF than OSM/LIF in terms of high expression in the brain (Fujiki et al., 2003). In goldfish, it was observed to share similar sequences (except the presence of signal peptide) and expression with CNTF but exhibited LIF-like functions (Hanington et al., 2007). We now have reason to believe that at the evolutionary standpoint, M17/MSH is actually more related to CNTF than to OSM/LIF.

The MSH orthologues appear to be secreted molecules because they contain signal peptides and are likely to be cytokine-like molecules because they show the classic cytokine motif i.e. having the mRNA-destabilizing pentamer AUUUA (Akashi et al., 21994) and the consensus polyadenylation signals (ATTAAA) (Sarudnaya et al., 2003). As mentioned, the Japanese flounder MSH is ubiquitously and constitutively expressed in numerous tissues except in the brain suggesting its involvement in various physiological processes in fish. In particular, it is highly expressed in tissues/cells that are involved in immuno-hematopoietic functions such as gills, kidney and PBLs. Such expression pattern is similar to LIF/OSM rather than M17/CNTF. LIF and OSM exert numerous

effects on the regulation, proliferation and differentiation of cells of various origins including macrophages, embryonic stem cells, blastocyts and neuronal cells, and induce acute phase protein production in hepatocytes (Huising et al., 2006). CNTF is a neurotrophic factor that influences the growth and survival of cells in the neuronal lineage (Ip et al., 1991), and can also induce acute phase protein production in hepatocytes (Huising et al., 2006). M17, which is expressed in brain, kidney and blood leukocytes in goldfish, and expressed only in brain and PBLs in carp, has been found to induce production of nitric oxide in macrophages, stimulate proliferation of macrophages, and induce differentiation of monocytes to macrophages (Fujiki et al, 2003; Hanington et al., 2007).

Also, *po*MSH is stimulated by polyI:C, an interferon inducer, by bacterial LPS and PG suggesting that the gene has a role against viral and bacterial infection in fish. Induction of *po*MSH by viral and bacterial agents, as well as its constitutive expression in immuno-hematopoietic tissues, indicates that the molecule has cytokine-like function. A number of Japanese flounder cytokines reported so far exhibits the same properties including Tumor Necrosis Factor (TNF), Interleukin-1β (IL-1β), CSF3 and a novel CC chemokine JFCCL3 (Santos et al., 2006; Hirono et al., 2000; Kurobe et al., 2005; Khattiya et al., 2006). Poly I:C has been reported to induce IL6 (Hurst et al., 2002) while LPS and PG can highly induce chemokines and cytokines (Fortier et al., 2004; Suda et al., 2002; Lorgeot et al., 1997; Wang et al., 2000). Studying further the function of MSH in relation to fish hematopoiesis and immunity could prove to be interesting.

5. Conclusions

Granulocyte colony-stimulating factor has recently been identified in fish. Little is known about where it is expressed or how it is regulated. Our results show that duplicate CSF3 genes are expressed in fugu and pufferfish, and that CSF3 is also involved in interferon-related mechanisms and pathways since it is regulated by poly I:C. Lastly, we attempted to produce a recombinant Japanese flounder CSF3 protein because availability of large

amounts of CSF3 would allow studies of its structure and function, and possible use in disease management .

The observed difference in structure and expression between IL11a and IL11b are linked remains to be confirmed. Also, complementation of function of both duplicates at the protein level within a species should be investigated further. Nevertheless, we can conclude that both IL11 paralogues in fish are 'functional' at the expression level and in a manner that is complementary to each other. This has implication to for example strategies on the development of vaccines against fish pathogens. Combinatorial use of these genes for therapy may prove to be more effective than used separately.

It is quite interesting that MSH and M17 genes are likely to be present only in fish. In addition to this, MSH expression is more similar to OSM and LIF even though structurally it is more homologous to M17 and to CNTF except for the presence of a signal peptide. This structural and functional attributes of MSH further confirms the pleiotropic and redundant nature of cytokines particularly of the IL-6 cytokine subfamily. This likewise adds to the complexity of this group of molecules, which in fish appears to be more diverse than in higher vertebrates, additional evidence supporting the "more genes in fish" theory (Ohno., 1970). The identification of MSH also complicates the suggestion in previous reports that M17 is an ancestral molecule to LIF and OSM, and that its function is ancestral to the divergence of CNTF and LIF, issues that require further studies.

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CHAPTER 3

Duplicated immuno-hematopoietic cytokines in teleost fish: correlated expression and structural divergence

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Duplicated immuno-hematopoietic cytokines in teleost fish: correlated expression and structural divergence

Abstract

The Whole Genome Duplication (WGD) that is thought to have happened early in the evolution of ray-finned fishes is said to be the reason behind the explosive diversity of the fish taxa, the highest among the vertebrate group with more than 23,000 member species. Duplicate genes (paralogs) in fish have been reported in publications and whole genomic sequences but the in-depth analysis of the relationship of their biological structure and function remains to be wanting. This is more evident in immune-related cytokine genes, such as the immuno-hematopoietic cytokines most of which are believed to be evolving rapidly. Our analysis confirmed that the 4 teleost IL6-cytokine genes were in duplicates, grouped as Type As and Bs at the subfamily level. The duplicates were found to show contrasting, constitutive expression in tissues between them. Type As, observed to be more structurally and phylogenetically related to the mammalian orthologs, hence appearing to be the "original" genes, are more involved in antibacterial responses. In contrast, Type Bs, which showed increased structural divergence, exhibited involvement in both antibacterial and antiviral responses suggesting that the duplicates are undergoing subneofunctionalization. We likewise discovered that the structure of Type As and Bs, while generally conserved, have point differences that correlates to the divergent gene expressions of the IL6-cytokines. Type Bs, in particular, possessed additional conserved cysteine residues that were strongly predicted to influence the disulfide binding patterns of the protein, and hence its function. These results are the first to be shown in rapidly evolving immune-related genes at the subfamily level in teleost fish and have important implications to the study of its immune system.

1. Introduction

Analysis of the genomic phylogeny of zebrafish (*Danio rerio*), fugu (*Takifugu rubripes*), green spotted pufferfish (*Tetraodon nigroviridis*), and more recently of medaka (*Oryzias latipes*) (Taylor et al., 2003; Christoffels et al., 2004; Jaillon et al., 2004; Vandepoele et al, 2004; Kasahara et al., 2007) have revealed hundreds of duplicate genes being co-orthologous to single copy genes in tetrapods confirming the tratraploidization (whole genome duplication event) that occurred within 300-450 million years ago after the divergence of the teleosts and sturgeons (Hoegg et al., 2004). Such genomic

duplication phenomenon has been thought to be responsible for the extreme diversity of the ray-finned fishes (actinopterygians) with more than 23,000 member species (as reviewed by Volff et al., 2005).

What happens to the genes (and the gene function) after duplication is still a matter of controversy (Hughes, 2005) and a number of models have been put forward to explain the phenomenon. The first hypothesis (nonfunctionalization) assumes that the duplicate gene, because of its redundancy, evolves into a pseudogene through degenerative mutation and is therefore eliminated (Ohno, 1970, Lynch and Cornery, 2000). Sometimes, Ohno (1970) believes, that the duplicate gene acquires a new function (neofunctionalization) by chance mutation and the other copy fulfilling alone the original function although this may be a very rare occurrence or never happens at all (Hughes and Hughes, 1993). Another hypothesis states that the duplicated genes divide the ancestral gene function (subfunctionalization) through complementary loss of function (Lynch and Force, 2000; Lynch et al., 2001). Such hypothesis is favored especially when explaining the retention of the duplicate genes in the genome (like in teleost fish) since deletion of either gene will be disadvantageous to the organism (Hughes, 2005). Still, another recent hypothesis called subneofunctionalization suggests that a large number of novel gene functions is acquired by rapid subfunctionalization of the duplicate genes followed by a long process of neofunctionalization (He and Zhang, 2005).

IL6-cytokines subfamily, which belongs to the hematopoietic cytokine family or class 1 helical cytokines because of their helical bundle structure (Bazan, 1990; Sprang et al., 1993), plays important role in various physiological processes including hematopoiesis, immunity, development among others (Heinrich et al., 2003). Its members, which includes interleukin 6 (IL6), ciliary neurotrophic factor (CNTF), leukemia inhibiting factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1), cardiotrophin-2 (CT-2), cardiotrophin-like cytokine (CLC) and neuropoitin (NP), shares a 4-α helical bundle structure and uses a common gp130 receptor for signaling. Granulocyte colony-stimulating factor (CSF3), another hematopoietic cytokine that is involved in neutrophil development, uses a specific CSF3 receptor but is structurally related to IL6, hence they

are also classified as an IL6/GCSF/MGF protein family (Structural Classification of Proteins, SCOP A.26.1.1).

IL6-cytokines in teleost fish have been reported previously as paralogs including IL6a and -b, IL11a and –b as well as a teleost –specific M17 and M17 homologue (MSH) (as reviewed by Huising, 2006; Hanington et al., 2007a, Hwang et al., 2007). Very recently, LIF has been reported in zebrafish based on cloning and expression analysis (Abe et al., 2007; Hanington et al., 2007b) but it is still unclear whether this molecule is an orthologue of mammalian LIF, OSM or CNTF, or an ancestral gene for these, or if it is actually a teleost M17 orthologue. The related CSF3 paralogs, on the other hand, has been well established in Japanese flounder, fugu and green spotted pufferfish (Santos et al., 2006).

Our studies of Japanese flounder CSF3, M17 honologue and IL11b have interestingly revealed marked differential gene expression patterns compared to their duplicates in other species (Santos et al., 2006; Hwang et al., 2007; Santos et al., 2008-*submitted*). While, hundreds of gene duplicates have been identified in teleost fish (Christoffels et al., 2004; Jaillon et al., 2004), some of which have already been reported to exhibit subfunctionalized activity (Braasch., 2006; Seiler et al., 2004; Smith et al., 2006; Souza et al., 2005), the relationship of the gene structure and function has not yet been investigated. Divergent expression, regulatory-motifs and coding-sequences between duplicated genes have been found to all increase with time but it is still unclear how exactly these factors interrelate (Li and Gu, 2005). This is especially true and important with teleost fish immune-related genes, which are now known to be rapidly evolving (Santos et al., 2006; Stein et al., 2007; Steinke et al., 2007).

In this study, we present evidence of a subfamily-wide gene expression divergence between duplicate genes in the IL6-cytokine subfamily in teleost fish, grouped as Type A and Type B based on structural and phylogenetic analysis. Distinct constitutive expression is seen between the duplicates for each gene in the subfamily. Furthermore, Type A genes were shown to be more involved in antibacterial responses, while Type B is involved in both antibacterial and antiviral responses. We also, for the

first time, show that such divergent expression, is correlated to the detectable divergent structure between the duplicate groups, wherein the Type B genes possessed additional conserved cysteine residues that are predicted to affect protein structures.

2. Materials and Methods

2.1. In silico gene and protein analysis

Sequence data for teleost fish IL6-cytokine subfamily members were collected from GenBank. The nucleotide sequence, translated amino acids, and average molecular weight were analyzed and determined using GENETYX 7.0.3 (GENETYX Corporation). SignalP (http://www.cbs.dtu.dk/services/SignalP/) was used to predict signal peptide cleavage. Identities were calculated using BLASTp (BLOSUM 62) implemented in BLAST 2 SEQUENCES (http://www.ncbi.nlm.nih.gov/ blast/bl2seq/wblast2.cgi) and the complete multiple amino acid alignments were carried out in CLUSTAL X 1.81 using default parameters.

To confirm paralogy, phylogenetic analysis was done using the Neighbor Joining (NJ), Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Minimum Evolution (ME) algorithms implemented in the MEGA3 (http://www.megasoftware.net/index.html/) employing the Poisson correction method with 1000 bootstrap re-sampling and with complete deletion of gap sites. Only the NJ bootstrap consensus tree was shown. The *Drosophila melanogaster unpaired* 2 gene was used as an outgroup.

2.2. Bioinformatic data gathering and analysis

Published RT-PCR expression bands for teleost fish IL6s, IL11a, CSF3s and M17/MSHs were stored and scanned using ImageJ software (Abramoff., 2004) and mean relative expression analyzed following Santos et al. (2006) with minor modification. Values were then plotted in a line graph and superimposed with a regression line using the Trendline option of MS Excel. The 3D protein structure prediction was carried out using Phyre (http://www.sbg.bio.ic.ac.uk/~phyre/) the successor to 3D-pssm (Kelly et al.,

2000). Disulfide bonds were predicted using a DiAmino Acid Neural Network Application (DiANNA): a unified software for cysteine state and disulfide bond partner prediction (http://clavius.bc.edu/~clotelab/DiANNA/) (Ferre and Clote, 2005), and by a cysteine disulfide bonding state and connectivity predictor DISULFIND, (http://disulfind.dsi.unifi.it/) (Ceroni et al., 2006). "Relative accuracy score" of the disulfide bong prediction were indicated, which in this study was qualified as very likely (predicted twice, +++); likely (predicted once, ++); unlikely (predicted but 1 or t C residues not conserved, +) and not possible (no prediction, 0).

2.3. Expression analysis/confirmation

RT-PCR expression for members of IL6-cytokine genes that have not been reported previously was conducted including for the Japanese flounder MSH *in vitro* stimulation in tissues following LPS and poly I:C treatment and CSF3 *in vivo* expression in kidney after *Edwardsiella tarda* and Hirame rhabdovirus (HIRRV) infection. Briefly, treatment concentrations used followed previous studies (Hwang et al., 2007; Santos et al., 2008-*submitted*) and sampling was conducted at 0, 1, 3 and 6 hrs pos-stimulation. Basically, total RNA was extracted from normal, treated and control tissues/cells using Trizol and cDNAs were synthesized using a cDNA synthesis kit. PCR conditions were: initial denaturation at 95^{o}C for 5 min, cycles of 95^{o}C - 30s, 55^{o}C - 30s, 72^{o}C - 1 min, and final elongation at 72^{o}C for 5 min. Normal and immunstimulated samples were ran at 30 cycles and 26 to 28 cycles, respectively.

3. Results

3.1. Evolutionary features of teleost IL6-cytokine subfamily

Teleost fish IL6-cytokines amino acid sequences collected/cloned and verified for analysis include 5 IL6, 9 IL11, 5 CSF3 and 6 MSH/M17 genes from various fish species reported (accession numbers reflected in Fig. 1). Phylogenetic analysis and multiple amino acid sequence alignment of these teleost fish genes, as compared with their

mammalian counterparts, confirmed their orthology and paralogy, being grouped to each gene clade, and the paralogs placed in each separate duplicate group (Figs. 1, 3A, 4A, 5A and 6A.). The paralog groups were collectively classified as Type A genes and Type B

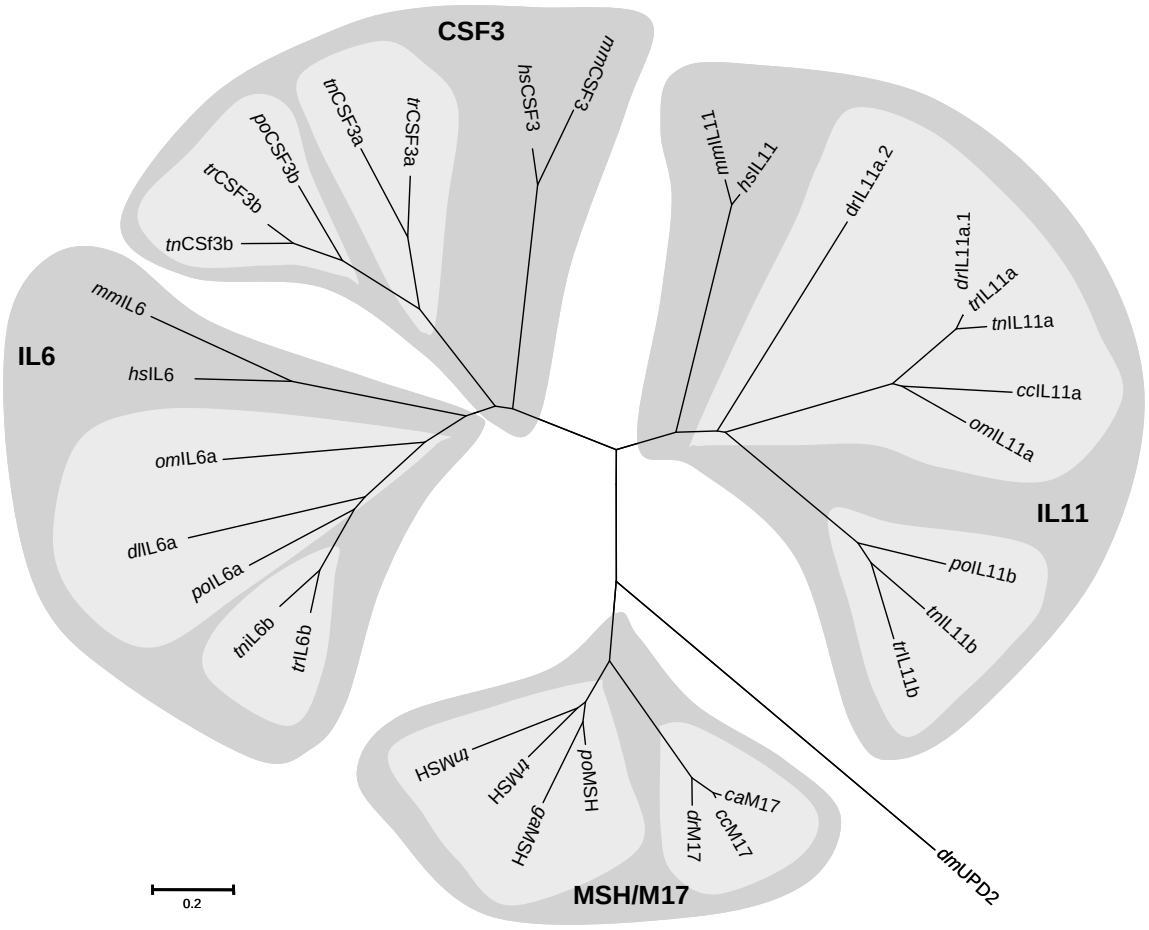

Figure 1. Neighbor joining tree of duplicated α-helical immuno-hematopoietic cytokines. Orthologs are shaded gray and paraloges in light gray. *Drosophila melanogaster* unpaired 2 (*dm*UPD2)-NP_573277, was used as an outgroup. Bootstrap was done at 1,000 resampling with complete deletion of gaps, and using the Poisson correction model. Abbreviated name and corresponding accession numbers : **Interleukin 6 (IL6)**: Human IL6 (*hs*IL6) –CAG29291, Mouse IL6 (*mm*IL6) –AAI32459, Rainbow trout IL6a (*om*IL6a) –ABI48359, European seabass IL6a (*dl*IL6) –CAM32185, Japanese flounder IL6a (*po*IL6a) –ABB90401, Fugu IL6b (*tr*IL6b) –CAD67609, Green spotted pufferfish IL6b (*tn*IL6b) –CAD88198; **Interleukin 11 (IL11)**: Human IL11 (*hs*IL11) –AAH12506, Mouse IL11 (*mm*IL11) –AAI34355, Fugu IL11a (*tr*IL11a) -BN000713, Green spotted pufferfish IL11a (*tn*IL11a) -BN000715, Rainbow trout IL11a (*om*IL11a) –CAI29480, Carp IL11a (*cc*IL11a) –CAG14936, Zebrafish IL11a.1 (*dr*IL11a.1) -BN000717, Zebrafish IL11a.2 (*dr*IL11a.2) -BN000718, Japanese flounder IL11b (*po*IL11b) -AB299205, Fugu IL11b (*tr*IL11b) -BN000714, Green spotted pufferfish IL11b (*tn*IL11b) –BN000716; **Granulocyte colony-stimulating factor 3 (CSF3)**: Human CSF3 (*hs*CSF3) –AF388025, Mouse (*mm*CSF3) –NM_00971, Fugu CSF3a (*tr*CSF3a) – Tr. Chr. Un, scaffold 571, Green spotted pufferfish CSF3a (*tn*CSF3a) –GSTENG00024099001, Fugu CSF3b (*tr*CSF3b) –SINFRUG00000157575, Green spotted pufferfish CSF3b (*tn*CSF3b) –Tn. Chr. Un, scaffold 13844, Japanese flounder CSF3b (*po*CSF3b) -AB200968; **M17 Homologue/M17 (MSH/M17)**: Japanese flounder MSH (*po*MSH) -AB280428, Green spotted pufferfish MSH (*tn*MSH) -CAF99247, Fugu MSH (*tn*MSH) –Ensembl: SINFRUP00000170397, Stickleback MSH (*ga*MSH) -Ensembl: ENSGACT00000022426, Carp M17 (*cc*M17) -AAM52337, Zebrafish M17 (*dr*M17) -XP_684795, Goldfish M17 (*ca*M17) -DQ861993.

genes. It was also found out that MSH falls under the Type A category and M17 as Type B. Succeeding NJ analysis showed that Type As, rather than Type Bs, were more closely related to its mammalian single gene copy counterpart and that Type Bs had diverged considerably from Type A/mammal cluster. Furthermore, the M17/MSH cluster was also shown to have significantly mutated from the other members of the IL6-cytokine subfamily and is more closely related to fly *unpaired* 2 gene. The relationship, however, of M17 and MSH to its mammalian counterpart was not possible as they are teleost fish-specific.

3.2. Comparison of relative gene expression in tissues

Our analysis of existing gene expression studies as well as our own RT-PCR experiments for Japanese flounder has revealed that there is discernible difference in the constitutive expression of the genes in tissues in different fish species studied so far (Table 1). IL6 Type A are expressed in spleen but not Type B. IL11 Type A is moderately expressed in brain, gills, kidney while Type B is highly up-regulated only in PBLs. CSF3 Type A, is not expressed in almost all the tissues checked but Type B is highly up-regulated in gills, kidney, PBLs and moderately in spleen. MSH (Type A), is highly induced in gills, kidney, PBLs and moderately in spleen, while M17 (Type B) is only highly stimulated in the brain and moderately in kidney and PBLs.

3.3. Comparison of relative gene expression in response to bacterial and antiviral agents

From the diverse data we collected, we narrowed our immunostimulation analysis on the kidney, peripheral blood leukocytes (PBLs) and spleen stimulated by bacterial (LPS and *E. tarda*) and viral (poly I:C and HIRRV) agents at 1 hr, 3 hrs and 6 hrs post-stimulation because this was the most constantly used. Surprisingly, when we checked and then plotted the cumulative mean relative expression of the duplicated groups, we found out that Type A was generally stimulated by bacterial agents constantly at each time-points but not by viral agents (Fig. 2). On the other hand, Type B gene expression appears to exhibit more activity in response to immunostimulants than Type As and that

Table 1. Comparative tissue expression of immuno-hematopietic cytokines. Assigned values of expression; +++ (high), ++ (moderate), + (low), - (non detected), nd (no data), collected from available data from published reports and from this study. Only tissues with available expression data for all 4 genes studied were selected for comparative purposes.

Gene	Type	Tissues							Source
		Brain	Gills	Kidney	Liver	Muscle	PBLs	Spleen	
IL6	a	-	-	+	-	-	+ +	+	Nam *et al.,* 2006 Iliev *et al.,* 2007
	b	-	-	+	-	-	nd	-	Seplucre et al., 2007 Bird *et al*., 2005
IL11	a	+ +	+ +	+ +	+	+	-	+	Wang *et al*., 2005 Huising *et al*., 2005
	b	-	-	+	-	-	+ + +	+	Santos *et al*., in prep
CSF3	a	-	-	-	-	-	+	-	Santos *et al*., M.S. thesis
	b	+	+ + +	+ + +	-	-	+ + +	+ +	Santos *et al*., 2006.
MSH/M17	a (MSH)	-	+ + +	+ + +	+	-	+ + +	+ +	Hwang *et al.,* 2007. *In Press* This study
	b (M17)	+ + +	-	+ +	-	-	+ +	-	Fujiki *et al.,* 2003 Hanington *et al.,* 2005

tends to increasingly induced by viral rather than bacterial agents. Furthermore, Type Bs interestingly showed marked up-regulation than Type As following viral induction but showed no significant difference in mean expression between Type A and B genes in response to bacterial agents. These significant differences in gene expressions can even be seen by the banding patterns alone.

3.4. Structural conservation and divergence

When ran in protein domain search/algorithms such as BLAST and ProDom, teleost fish IL6, IL11, CSF3 and M17/MSH orthologs/paralogs have been identified to be homologous to the archetypal IL6 domain i.e. their important domains were predicted to be conserved with IL6. These were likewise predicted by 3D modeling server Phyre at over 90% Estimated Precision Values showing the general 4 α-helical bundle structure trained on known crystallographic protein folds. Interestingly, M17 and MSH, which do not have a mammalian orthologue, also were predicted to have a 4 antiparallel α-helix structure.

Multiple alignment data analysis, however, revealed conservation-disappearance of C residues across the IL6-cytokine orthologs (Figs. 2A, 3A, 4A and 5A). For IL6, of the 4 important C residues in mammals, only the 3rd and 4th C residues are conserved in teleost fish IL6 orthologs. Moreover, between the IL6 duplicate groups, Type B genes were composed of an additional C residue at the C-terminal, Helix D region of the protein. For CSF3, almost similar to IL6, of the 4 important C residues, only the 4th C residue is conserved in teleost fish. We, likewise observed conservation of C residues in Type B but this time, there were 2, located at the IL6 signature motif and in Helix D. IL11 orthologs in teleost fish, which in mammals do not possess structurally important C residues, contained one conserved C residue located in the Helix D region and Type B, again having 2 conserved residues at the N- and C-terminal ends of the mature IL11b proteins. With M17 and its apparent paralogue, MSH, which are believed to be only found in teleosts, we observed 4 conserved cysteine residues, and like IL6, IL11 and CSF3, we again found 2 extra conserved residues in M17s, the Type B genes for this group.

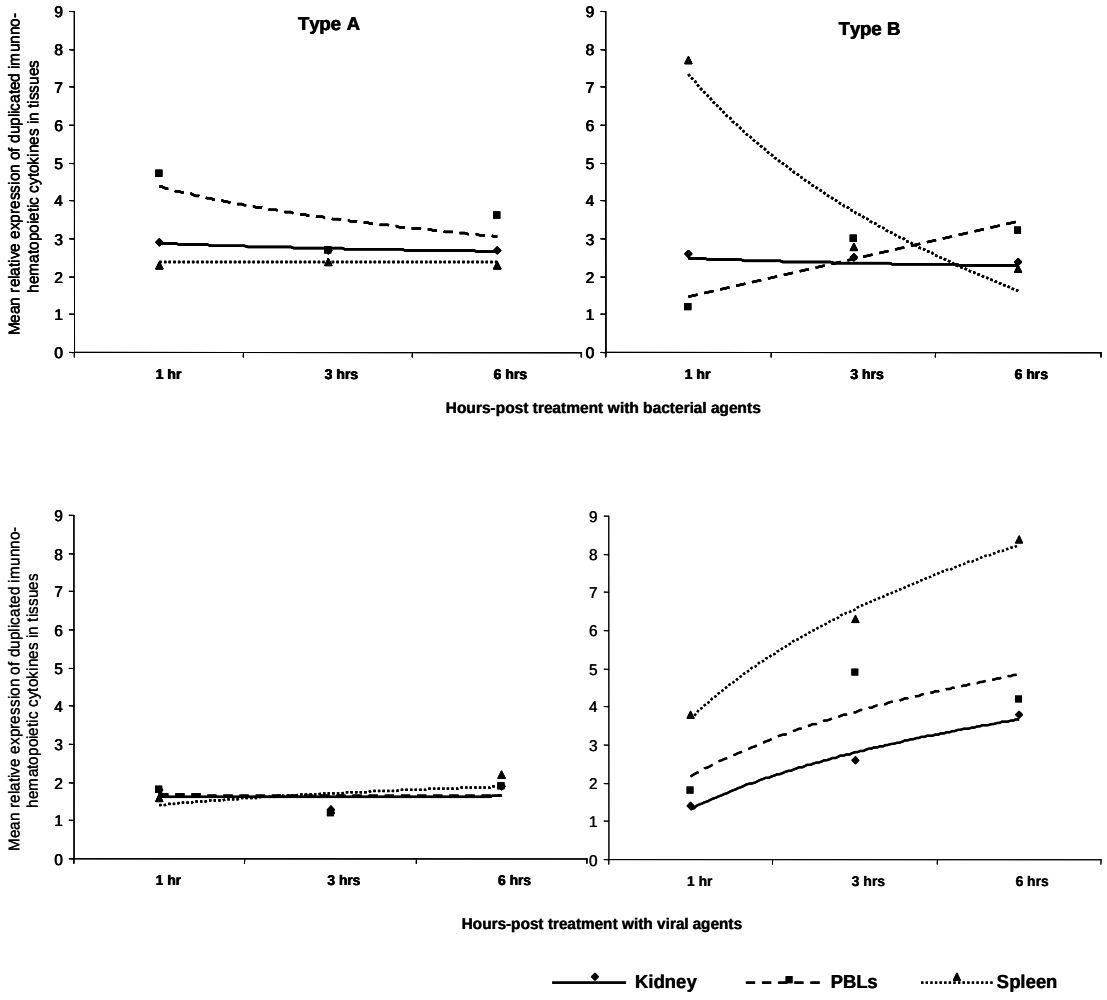

Figure 2. Mean *in vitro* expression levels of type a and type b immuno-hematopoietic cytokine genes in tissues. A) In response to bacterial agents such as lipopolysaccharide (LPS) and bacterial infection at 0 hr to 6 hrs post-treatment; and B) In response to viral agents such as polyI:C (a viral mimic) and viral infection at 0 hr to 6 hrs post-treatment.

To check whether these conserved C residues could possibly affect the structures of proteins they belong to, we performed prediction analysis of disulfide connectivity. Results revealed that the disulfide bonding patterns tends to be consistently predicted in Type As by both prediction models in contrast to Type Bs, especially if the non-conserved C residues for *tr*IL11a, *tn*IL11b and *tn*CSF3a were not considered (Table 2). By way of 3D modeling, we were able to confirm the placement of cysteine residues in the predicted tertiary fold for each representative protein (Figs. 2B, 3B, 4B and 5B).

Using this, and the disulfide prediction models, we show 3D structural differences between Type A and Type B genes. For IL6 Type A, the 2 conserved residues were clearly forming disulfide bonds. With Type B, however, the presence of a 3rd C residue in Helix D can possibly alter the binding connectivity of the 1st and 2nd C residues, such that base on prediction, there is a high probability that the 2nd and 3rd residue can also bind (broken white line). For CSF3 Type A, only 1 conserved free C residue is observed while for Type B, the additional 2nd and 3rd C residues in Helix C and Helix D, respectively can possibly bind (white broken line). IL11 Type A, like CSF3 Type A, is composed of only 1 free C residue found in Heix D. The 2 conserved C residues of IL11 Type B, however, which are both found in Helix D, could also form a disulfide bond that is predicted to distort/bend the C-terminal part of Helix D. Finally, prediction models clearly show that the 3rd and 5th conserved C residues in M17 and MSH form disulfide bonds. Specifically for M17 (Type B), the 4th conserved C residues can form bonds the 1st and 2nd conserved residue (white broken line).

The respective phylogenetic grouping between and among mammalian and teleost fish IL6-cytokines in this study confirms their clear orthology as well as their paralogy. Type As, as shown also by comparative analysis, are more related to mammalian single copy orthologues than Type Bs indicating that the former are the original genes and the latter are the paralogs. Previously, we have shown by gene and synteny analysis that the teleost CSF3as are indeed the ancestral gene copies and the CSF3bs are the duplicates (Santos et al., 2006). Furthermore, we showed that the duplicate genes are undergoing asymmetric accelerated evolution with Type Bs diverging more rapidly than Type As. Such type of duplicate gene fates has recently been found to be not uncommon in teleost fish (Steinke et al., 2006).

Table 2. Predicted cysteine residue connectivity (disulfide bonding) in teleost fish IL6- cytokines. Relative accuracy score is qualified by the following: very likely (+++) predicted twice; likely (++) predicted once, unlikely (+) predicted but not conserved; no binding (0) no prediction.

Gene	Paralogs	DiANNA		DISULFIND		Relative accuracy score
		C^n - C^n	Cn-Cn	C^n - C^n	Cn-Cn	
IL6	*po*IL6a	C^{72}-C^{82}	C1-C2	C^{72}-C^{82}	C1-C2	+++
	*om*IL6a	C^{83}-C^{91}	C1-C2	C^{83}-C^{91}	C1-C2	+++
	*tr*IL6b	C^{81}-C^{174}	C2-C3	-	-	++
	*tn*IL6b	C^{71}-C^{170}	C1-C3	-	-	++
IL11	*cc*IL11a	-	-	-	-	0
	*om*IL11a	-	-	-	-	0
	*tr*IL11a	C^{115}-C^{157}	Cx-C2	-	-	+
	*tn*IL11a	-	-	-	-	0
	*dr*IL11a.1	-	-	-	-	0
	*dr*IL11a.2	-	-	-	-	0
	*po*IL11b	C^{12}-C^{175}	C1-C3	-	-	++
	*tr*IL11b	C^{158}-C^{174}	C2-C3	-	-	++
	*tn*IL11b	C^{1}-C^{158}	Cx-C2	-	-	+
		C^{10}-C^{174}	C1-C3	-	-	++
		-	-	C^{158}-C^{174}	C2-C3	++
CSF3	*tr*CSF3a	-	-	-	-	0
	*tn*CSF3a	C^{27}-C^{160}	Cx-C1	C^{27}-C^{160}	Cx-C1	+
	*tr*CSF3b	C^{80}-C^{165}	C1-C3	-	-	++
	*tn*CSF3b	C^{88}-C^{165}	C2-C3	-	-	++
	*po*CSF3b	C^{90}-C^{168}	C2-C3	-	-	++
MSH/ M17	*po*MSH	C^{13}-C^{50}	C2-C3	C^{13}-C^{50}	C2-C3	+++
		C^{157}-C^{182}	C5-C6	C^{157}-C^{182}	C5-C6	+++
	*ga*MSH	C^{13}-C^{182}	C2-C6	-	-	++
		C^{50}-C^{157}	C3-C5	-	-	++
	*tr*MSH	C^{13}-C^{49}	C2-C3	C^{13}-C^{49}	C2-C3	+++
		C^{156}-C^{181}	C5-C6	C^{156}-C^{181}	C5-C6	+++
	*tn*MSH	C^{15}-C^{170}	C2-C6	C^{15}-C^{170}	C2-C6	+++
		C^{48}-C^{147}	C3-C5	C^{48}-C^{147}	C3-C5	+++
	*cc*M17	C^{4}-C^{152}	C1-C5	-	-	++
		C^{9}-C^{178}	C2-C6	-	-	++
		C^{45}-C^{117}	C3-C4	-	-	++
	*ca*M17	C^{4}-C^{153}	C1-C5	-	-	++
		C^{9}-C^{118}	C2-C4	-	-	++
		C^{153}-C^{179}	C5-C6	-	-	++
		-	-	C^{9}-C^{179}	C2-C6	++
	*dr*M17	C^{4}-C^{178}	C1-C6	-	-	++
		C^{9}-C^{153}	C2-C5	-	-	++
		C^{46}-C^{118}	C3-C4	-	-	++
		-	-	C^{9}-C^{178}	C2-C6	++

C^n : Cysteine position
Cn: Conserved cysteine number
-- : No prediction

4. Discussion

The differential expression patterns between IL6-cytokine gene paralogs in tissues suggest that they are both functional at least at the transcription level and their involvement is divergent. The function of the paralogs become clearer following immunostimulation studies, where it was shown that Type As responds more to bacterial agents while Type Bs responds well with viral agents. The propensity of Type A genes to be involved in antibacterial responses is similar to its mammalian orthologues. For example, human CSF3 and IL6 are induced by LPS (Sallerfors et al. 1992). This is not surprising as Type As have been shown to be the ancestral genes by phylogenetic analysis. It is believed that the innate immune function, where antibacterial responses dwell, is more primitive than adaptive immunity where antiviral responses are considered (Janeway and Medzitov 2002). On the other hand, the clear role in antiviral responses of Type Bs suggests that it had gained a novel function. This gain of function per se is also no longer surprising in teleost fish paralogs because increasing evidence have now shown such phenomenon. For example, a macrophage colony stimulating factor (CSF1) receptor paralogs evolved as co-evolutionary units and that the B-paralogons showed a higher

Figure 3. Multiple alignment and corresponding predicted tertiary structure of Interleukin 6 (IL6) orthologues and paralogues. A) Putative signal peptide (black inverted triangle), signature motif (shaded gray) conserved cysteine residues (shaded black) and conserved cysteine residues for fish IL6b only (shaded red) are indicated in the multiple alignments. B) Important putative functional elements such as possible cys binding and motif are indicated by white circles in the predicted tertiary structures.

Figure 4. Multiple alignment and corresponding predicted tertiary structure of Interleukin 11 (IL11) orthologues and paralogues. A) Putative signal peptide (black inverted triangle), signature motif (shaded gray) conserved cysteine residues (shaded black) and conserved cysteine residues for fish IL6b only (shaded red) are indicated in the multiple alignments. B) Important putative functional elements such as possible cys binding and motif are indicated by white circles in the predicted tertiary structures.

Figure 5. Multiple alignment and corresponding predicted tertiary structure of Colony stimulating factor 3 (CSF3) orthologues and paralogues. A) Putative signal peptide (black inverted triangle), signature motif (shaded gray) conserved cysteine residues (shaded black) and conserved cystein residues for fish IL6b only (shaded red) are indicated in the multiple alignments. B) Important putative functional elements such as possible cys binding and motif are indicated by white circles in the predicted tertiary structures.

Figure 6. Multiple alignment and corresponding predicted tertiary structure of M17 homologue/M17 (MSH/M17) orthologues and paralogues. A) Putative signal peptide (black inverted triangle), signature motif (shaded gray) conserved cysteine residues (shaded black) and conserved cystein residues for fish IL6b only (shaded red) are indicated in the multiple alignments. B) Important putative functional elements such as possible cys binding and motif are indicated by white circles in the predicted tertiary structures.

```
HumanIL6    -MNSFSTSAFGPVAFSLGLLLVLPAAFPAPVPPGEDSKDVAAPHRQPLTSSERIDKQIRYILDGISALRKETCNKSNMCE
MouseIL6    -.KFL.ARD.H....-...M..TTT...TSQVRRG.FTEDTT.N.PVY.T.-QVGGL.THV.WE.VEM...L..GNSD.M
poIL6a      -.A.KHNADLSSA.MLAA...CALG.PVEYE.TDSPAG.FSGEEQEVTPDLLSASPVWDL.IGVTAHHQ..FEDEFQQEV
drIL6a      --------------------------------------------------------------------------------
omIL6a      MNS.TRYLSLLSALVV.VKGNPV.S.LAELMTS.WT.GEELGTDGETGAPPKWEKMIKMLVHEVTTLRNQQFVEEFQKPV
trIL6b      -.A.I.--YLLAPLVLAAV.QPTAG.PLD-A.TESPAGETSGEEAETGSPDDALAVALESV.GATKLHKN.FLVEFQGEV
tnIL6b      -.SL.T--NLL.PLLLAAA.RLAAG.PLE-G.TESPAGETSGEELETGRPEDALAVALESV.RATKRHK..FLAEFQGEV

HumanIL6    SSKEALAENNLNLPKMAEKDGCFQSGFNEETCLVKIITGLLEFEVYLEYLQNRFESSE-EQARAVQMSTKVLIQFLQKKA
MouseIL6    NNDD.......K..EIQRN..Y.T.Y.Q.I..L..SS....YHS....MK.NLKDNKKDK..VL.RD.ET..HIFNQEV
poIL6a      KYR---FL.HYK.SSLP--AD.PSAN.SK.A..QRLAE..HTYM.LFKHVEKEYP..SILLHARYHSGAL--.GLIKE.M
drIL6a      ------------YSLT-SKT.PEKS-SK.A..RCLAQ...TYTAL.KHVEKESP..IRSEGHSFKSLLLR.TSGIKN.M
omIL6a      EEISSFSQHQVPSTPPHLSKTLC.AS-.K.A..QE.SR..QVYQLL.QHVKAEYPQ.TLLPSVTH.TTVL--.GLVKDQM
trIL6b      KYD---FLDRYKI.SLP--AK.PY.N.GKDA..RRLLE...IYS.L.KRVEEE.P..SILSEVRFYSNIL--.KE.EN.V
tnIL6b      KYE---FLDRYKI.SLP--AK.PY.N.GQVA..RRLLE...VLLKHVVYSVLLKH-GS-LSEVRYYSNVL--.KEVEN.V
                                   1         2
HumanIL6    KNLDAITTPDPTTNASLLTKLQAQNQWLQDMTTHLILRSFKEFLQSSLRALRQM--------------------------
MouseIL6    .D.HK.VL.T.IS..L.TD..ES.KE..RTK.IQF..K.LE...KVT..ST..T--------------------------
poIL6a      R.PGQV.V.TSRQEQQ..QDMDNPSTFHRK..A.N...QLHN..RNGKV.I.KREMPKQKRRKDDGIIPPIHPSYQMT--
drIL6a      RHREHVKALTNSQEGH..RDFDSPDPFQRL...FK..YKLRD..IDGIKNIIKK---TIVWPMGVRYRYTTNNYKSITNQ
omIL6a      .VAEVVEDLSASERKRV.GEVSTGTE.ERKTSV.A...ELRN..VDTK....R.G------KRGKDFQ------------
trIL6b      RDR.QVMRLTSSQEEQ..KDTDYPDTFHRK..A.G..YNLHY..VDCR.VINKR--AKHRESAGSRVVRAVTFYHPKKRS
tnIL6b      .ERGQV..LSSSQQEQ..RAVDRSDTFHRR..A.G..YNLHY..VDCR.VIVN-----KRARAG-RALAPVTFQQRLTT-
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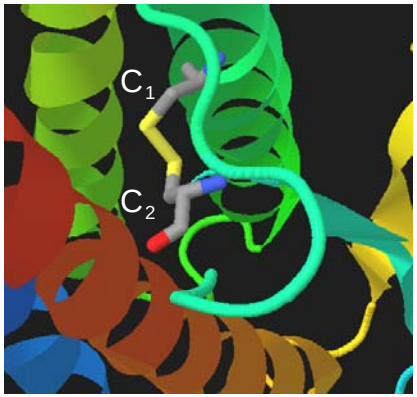
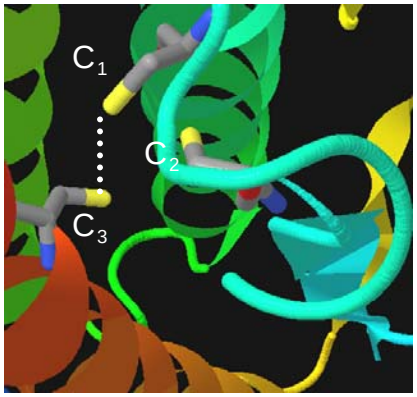


Japanese flounder IL6a



Fugu IL6b

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HumanIL11   ---MNCVCRLVLVVLSLWPDTAVAPGPPPGPPRVSPDPRAELDSTVLLTRSLLADTRQLAAQLRDKFPADG--DHNLDSLPTLAMS-AGALGALQLPGVLTRLRADLLSYLRHVQWLRRAGGSS
MouseIL11   ---.................RV......A.S....S....D...A.................M..........-..S..........-..T..S.............V..M..............P.
trIL11a     MKLLLDSSSSL.FS.L.AQLPVFVSAS.VPHR.P-----SDM.RLSNQ.KH.MKL.---QEL..EHSFDSDVEP.RFT...EMSNRS.HS.NN.E.KPT.SQ.H...KL.EH.FE..N.VSKKH
tnIL11a     MKLLLDSSSSL.FS.L.AQLPLLTSTS.VPHR.P-----SDM.RLSNQ.KH.IKL.---HEL.K.HSFDSDVEP.KFP...EMSNRS.ND.NN.E.KPT.SQ.H.E.KL.EH.FE..N.VSKKH
omIL11a     MKLLVDSSSSL.LS.L.AQIPLFTSAV.APYR.PNV--VH...RLANQ.KN.RQI.----.DL.KEHAFETDPEQ.RFK...LMNNR-.SDINS.EMRPT.SQ.H...K.FEH.FA..S..SRKH
drIL11a.1   MKLLGDSSSSL.LS.L.AQLHLL.SAF.VHHR.NQ----IDF.KLSNQ.KL..TL.---RNL.K.RVFSTEINH.RFK...AISSR-.SDFAT.EVKPT.SQ.H.N.K.FQH.FE..NNITHKQ
ccIL11a     MKLLGDSSSSL.LS.L.AQLHLL.SAF.AH.R.IQ----TDF.KLSNQ..H..KL.---QDL.KNPVF.TEIDHQRFK...AISSR-VSD.TT.EFKPT.SQ.Y...K.FEH.FE..N.TTRKQ
drIL11a.2   MKLSPDSTFPLIILMACVELFDFIRAR.ANL.QGK----KH.STLYQDM.M..KL.-----SQQMNANELTDFE.S.S...S.NY.-VKD.HS.EVSST.AQ.YSG.K.FKF.LD.VQ.NSDE-
trIL11b     MDVIEDSAPCL.HLFL.AELIVHSASR.ASS.APC----RTFR.IFHQVDL.MGLS.K.HDLSDEDVLMFESMENR..T..H.PH.-.EYFRSFKVNES.SQ.SLHTQ.FRQ.ID..KL.RENV
tnIL11b     MKLIDDSAPNL.CL.L.AELIVHSTCR.A.GAALC----TN.K.MLHQVDR.IGLSSK.HGLSDEEVLIIARMENS.....H.QH.-.EYFRS..VNESFSQ.YLQTE.FRQ..D..K..QDNC
poIL11b     MKLLHDSIPCLFHL.L.AELFVPSSSR.VHTSSLC----RMFG.MIHQVDK.TDISKN.HELSDNNELLNS-A.NK.PD..HMQH.A.HFFNS.KMNES.SE.YLLAQAFRL..D..KTEKDNF
                                                1

HumanIL11   LKTLEPELGTLQARLDRLLRRLQLLMSRLALPQPPPDPPAPPLAPPSSAWGGIRAAHAILGGLHLTLDWAVRGLLLLKTRL----------------------
MouseIL11   .........A.....E................AA..Q.VI..G..A....S.....................----------------------
trIL11a     HHPAL.K.VEMIKEMKS.ITL.HCQ.L.VEA.RLT.AT.SL.P-HLPYQFDVLQSS.EL.QHFK.FC...Y.AF.S..PKVN-AAVQ----------------
tnIL11a     HHPAL.K.VEMIKE.KS.ISL.HHQ.L.VEA.RLNLTT.SL.P-QLPYQFDVLQSS.EL.QHFK.FC...Y.AFIS..PKV--SAVQ----------------
omIL11a     HHPAL.K..QMMSLIKS.TSM.EHQ.M.VDAQRLS.PS.SM.P-..P.QFDVLQSSQEL.LQFR.FC...Q.VFSV.S.KSKMSAVQ----------------
drIL11a.1   QHS-I.K.TDMVSHIGG.VNS..RQ.NHIGA.RL.VPS.SL.P-I.AFH.EMVQTSQEL.EQFS.FC...A.V.GRTRSL.TSREAPVVGSTGTSPSGPIRIVGK
ccIL11a     QHSSV.K.TDMISHIKS.INS..RQ.T.AEA.RI.VPS.SL.P-N.AFH.EVVQSSQEL.QQFR.FC...S.VF.T..SK.PA----------------------
drIL11a.2   ---.GNDYSKTKKIVHLIQAII.KVLQE.GQTA.EIVH.TL.P--LETF.QLYQTNAE.HKK.LIFC.YYT.A.GS..RKHPDTPS----------------
trIL11b     SLPSRAAE-DSSTH.LK.SNL.NASLHQMNEEV.QLP.LSL.--IA.TSFDVLQFSVE.SDR.KIFCH.SK.V.RY.QRLNRCPKH----------------
tnIL11b     SLPSQAAR-SSS.H.LQ.SKL.DASLQQMDQAV.QAPL.SF.--VV..SFEVLQFSVE.SDQ.TVFC...TK.V.RAVQRVSRCPTPTLRLRG----------
poIL11b     SLPSQSAE-DASTH.LQ.SNL.NMSLHQMSAET.Q.PA.SL.--VV...FDLLQFSIE.SER.KVFCN.SK.V.RS..-LPRCRRQ----------------
                                                                                2                 3
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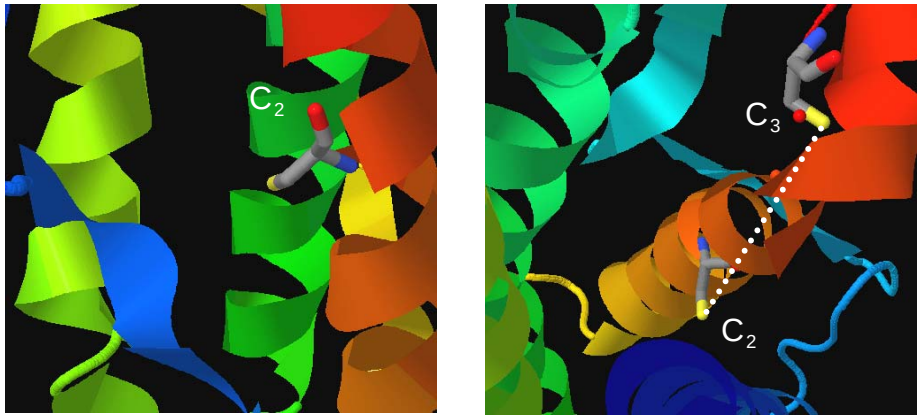

Fugu IL11a **Fugu IL11b**

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HumanCSF3   MAGPATQSPMKLMALQLLLWHSALWTVQEATPLGPASSLP------QSFLLKCLEQVRKIQGDGAALQEKLCATYKLCHP
MouseCSF3   ..QLSA.RR..........Q....SGR..V..VTV.A..PSLPLPR.....S........AS.SV.L.Q.........
trCSF3a     ------MNILIVLVIPYMAMLGCGAP.PGSSA.VEDPQTQELVQTSRLL.Q.V.MAIPETHRSSVQSEVRQESSL..NSS
tnCSF3b     ------MHILIVLV.PYMAMLGSGAP.-------------DLLLSSR.L.A.IQNAIPFTHSACVQSE-----SL..NSS
trCSF3b     -----MTDLTV.LL..YF.FV--VQSAPVGP.-E.TPD.TDVAERARTLVQ.I.RDIPVAH.AAISTK-----GLT.DSA
tnCSF3b     -----MIHLTV.LL.HH.PPA--VRSAPVGSA-DLTLD.TDVAEPARTLVQ.I.KDIPVAHAVAVSSR-----GLT.ESS
poCSF3b     -----MD.ETVVAL.YYF.FAVLVQS.PISPAPNTPPV.KEAAERAKTLVE.I.RELPAVHTATVNTE-----GLT.DPA

HumanCSF3   ---EELVLLGHSLGIPWAPLSSCPSQALQLAGCLSQLHSGLFLYQGLLQALEGISPELGPTLDTLQLDVADFATTIWQQM
MouseCSF3   ---.............K.S..G.S.....QTQ.........C.........S....A.A....L......N.........
trCSF3a     -ENTK..IMASTI...P..VLKAL.ENFTMGT..RRISE..Q.HRT..AVIADHLKNKD-RVLA..A.IR.LNIQ.NKML
tnCSF3a     -ENSKYEKMASII...A..VLKAL.PNVT.ETS.ALVSK..Q..ED..GIIVNHLEQKK-E.SD.KAHIS.LKKL.TRML
trCSF3b     -QPTN.QVMSL...L.V...LKP..EQFT.DI.V.RMLV.CQMF.K..GV.SERVDG----.MD.KVTLR.LV.H.TKMT
tnCSF3b     -QPTN.Q.MTE...L.V...LKL..DHFT.DM.V.RMLV.CQMF.R..AV.SEKLDG----.MD.KVTLR.LV.H.TKMK
poCSF3b     PQTPN.QMMVT.....AT.ILKPL.ERFTMDM.V.RMSV.CL.....GV.ADRLSG----.TN.RA.LR.LL.H.NKMK
                                              1       2

HumanCSF3   EELGMAPALQP---TQGAMPAFASAFQRRAGGVLVASHLQSFLEVSYRVLRHLAQP--------
MouseCSF3   .N..V..TV..----..S.....T..........AI.Y..G...TARLA.H...----------
trCSF3a     KMV.EETVVP.-----AVTLNLPADYEVQVAAH.TLQQ..T.GRDVD.H.KS.DKTVDEEPDDR
tnCSF3a     KVA.GQAEDL.-----KPTLNLPGDYEVQVAAH.TLLQ....GQDVG.C.ES.D.SR-------
trCSF3b     .TVRLNGDTPE-APSWD.ASRLPGNYEAQMAAH.TLIQ.R..CHDLT.S..AISTYRTSAA---
tnCSF3b     .T..LDVDGSE-AL.VDVASRLHGDYEAQMAAH.ALVQ.R..CHDLT.S..AINSYRSSTA---
poCSF3b     .AAQFGAESPDQNQSLDLASRLHGNYEVQVAVHVTLTQ.R..CHDLI.S..AI.TYRRRAAGAR
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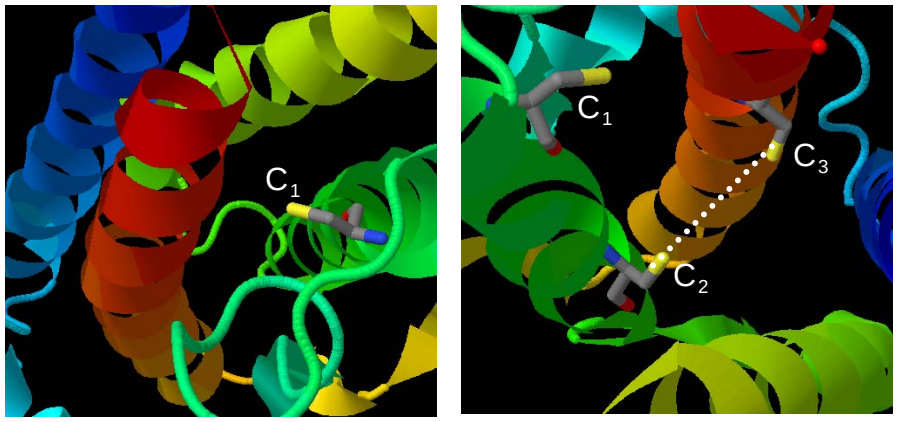

Fugu CSF3a **Fugu CSF3b**

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poMSH   MNGYVKRMSFQQFMELTTTLLSLLLVMAVDSTRTVAVSGSQQ--CGNSVQQTLKLTRLLKKESVDLIETYKASQGEMSED
gaMSH   ...H..SLR..P...PA.............S....ATRN..--.E..L...WRIA..VQ....E..K...T....NA.L
trMSH   ...H..SVHL.LX..AAAA..C.R.AV.....GG.MA..A.D--.A-L......I...VH...A...K..........F
tnMSH   ...H.RS.Q..RQ.KAAAL..C.W......A..STTA..K.AGD.----SRA..I.EV.L...E...KI..S...Y...L
ccM17   -----MVCLS.RSQAKFRMI.AI.ILI..ELVHPTVSCKNET--.SQLLRHS.R....MS.RTTE.L....T..-DFADL
caM17   -----MVCLS.RSQATFRMI.PV.ILI..ELVHPTVSCKNES--.SQLL.HS.R....TS.RTKE.L........DFADL
drM17   -----MLCLS.RLQVKFRAYFAIIILI..QLVQPTMSCKNEN--.SQRLHRS...NKFTN.IT.K.LD.......DSTDL
                                                  1       2

poMSH   LCNLSVNNIPDPNISGLEPSERIASIYTHLQAFLTHFKRVYEQQTDLQLPSNPLLAELTNVSTRSRNLASLINSFYQSLF
gaMSH   ..KA.F.Y..........W..LL...K...D.FP..S......M....S.ASR.....NRTNGGR....A..S.S.....
trMSH   F.KV.PSD..V.......Q.D.....S.Q.R..FP..R......S...P.TSA..SL.DV.RG.N.....VV..L....Y
tnMSH   R.KMPQS.V.N...A.......M...S......FP..R..H...S...P.TS--S.RAQR..GQGHQ.P------VPAPL
ccM17   I.DMQMD.V.VSTV..QTI.Q..L.V....KE..P.M.T.M...K..NP.T..VAEG.NRMI.HV.HI.VRV.CILEI.Q
caM17   I.EMQLD.V.VSTV..QTI.Q..L.V....KE..P.M.T.M......NP.T..VAEG.NRMI.HVTHI.VKV.CILEF.Q
drM17   I.EMQMD.V.VST...QTE....L...S..K...P.L.T.M...R..DP.T..VTEGINSLI.HV.HM.VRV.CLL.I.Q
         3                                                                        4

poMSH   PNLPMPEPAGGPTTLPPPQNVFQQKVYGCAVLKTHLEFLSNVYRELRTLKSKV-CRGI--------
gaMSH   ................S...........Q....YR.LV...C...K.M....-..SRKPVN----
trMSH   ....G.G.K.A..KP..R..I........V....YK......M..I.M...N.-.A.RMKRNVQLF
tnMSH   .QPAGAPAR....K.....Y.....I...V..N.YKN.....KS..N...K---.P----------
ccM17   ..I.I....ES..GI.HA..I....A...I..TRLQ.L..QAVQ.QKS..KGKM.RKSTKNGS--
caM17   ..I.I....ER..GI..A..I....A...I..TRLQ.L..QAVQ.QKS..KGKM.RKTTKDGS--
drM17   ..I.I....ER..GI..A..I........I..TRLQQL..QAVQ.QKS..-GKT.RRTKKNYS--
                                      5                    6
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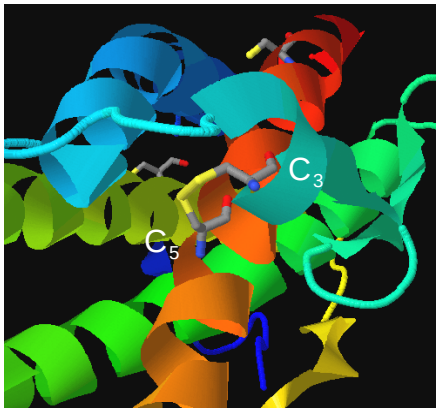

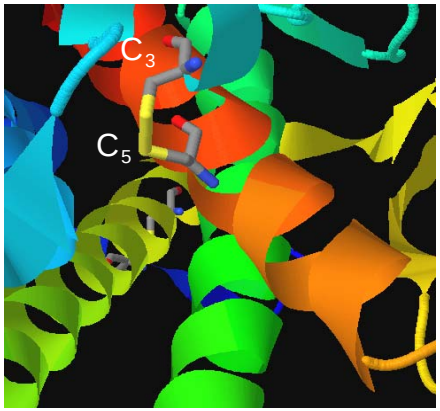

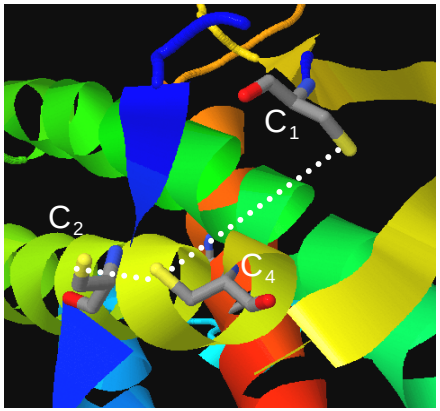

Fugu MSH **Zebrafish M17**

evolutionary rate that was implicated to have caused functional divergence of the extracellular, ligand-binding region of this receptor (Braasch., 2006). What is surprising though is the apparent retention of antibacterial as well as gain in antiviral roles for Type Bs suggesting that this highly mutating, immune-related gene subfamily is undergoing subneofunctionalization (He and Zhang, 2005). It would then be very interesting to learn whether this phenomenon indeed happens at the protein level.

Functionally important C residues were surprisingly only partially conserved in teleost fish IL6 and CSF3 orthologs and that potential disulfide bonds exist in teleost fish IL11 orthologs. The 4 conserved cysteine residues of mammalian IL6 and CSF3 proteins are crucial for their stability (Somers et al., 1997; Hill et al., 1993) while IL11, does not possess disulfide bonds, but maintain its 4 α-helical bundle structure using other covalent bonding mechanisms (Czupryn, 1995). Disulfide cross-link, which is produced by binding of 2 C residues, plays an important role in enhancing the thermal stability of especially soluble proteins to function properly in the oxidizing extracellular environment (Petersen et al., 1999). C residues in proteins exist in proteins as free cysteine (Cys_SH) and disulfide-bonding cysteine (Cys_SS), both of which behave like strongly hydrophobic residues (Nagano et al., 1999). Cys_SH is both active in metal binding and disulfide bond formation while Cys_SS is more for disulfide bond formation only. The partial conservation of C residues in teleost IL6 and CSF3 and appearance in IL11b suggest that they use different binding modules to stabilize their tertiary structures. In addition, the definitive disulfide bond prediction in Type A than Type B cytokines is apparently influenced by the additional conserved cysteine residues in the latter. This again suggests that structurally, Type As and Bs possess different tertiary conformations, which would likely affect protein function. It also possible that the Type Bs have significantly diverged from the Type As (the original genes) that they now exhibit slightly different tertiary structures, making it more difficult to be detected by the prediction models anchored on existing protein data, most of which are from mammals.

The appearance of extra C residues conserved among teleost fish Type Bs which appears to be correlated with antiviral responses at a subfamily level were quite intriguing. One possible explanation of the molecules' ontogeny is their common origin, being a group

thought to be monophyletic (Bazan 1990). However, how this occurred, especially amidst the history of major and minor chromosomal events in teleost fish evolution, needs further clarification. Moreover, we believe that these extra conserved C residues, based on our findings, play important contributory roles in the divergent expression patterns of the teleost fish IL6-cytokine paralogs. C residues that have been found to be conserved in a protein have been considered as catalytic residues (Ota et al., 2003) and in this study, the extra C residues were either involved in disulfide binding or are buried in the hydrophobic core suggesting its importance in the stability of the protein. Moreover, some of these extra C residues like in IL11 are placed in a location for primary receptor binding site, Site I (Czupryn et al, 1995) and could therefore produce a different binding pattern as compared to the Type A proteins. This, however, needs further studies.

Another interesting observation in this study is the predicted disulfide binding patterns in the teleost fish-specific M17/ MSH tertiary structure suggesting that these are important functional elements of the proteins. Like in IL6, and CSF3, these disulfide bonds are expected to provide structural stability to the M17/MSH proteins. Such analysis has been made possible by the prediction models used in this study and shows that these algorithms are very good, cost-effective tools in studying fish protein structures, as alternates for x-ray crystallographic or NMR analysis, which are more suitable to be used in human studies because of cost.

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CHAPTER 4

Discovery of a novel hematopoietin cytokine receptor from teleost fish involved in the Jak/STAT signal pathway

Keywords: Japanese flounder (*Paralichthys olivaceus*); type-1 cytokine receptor; novel; poly I:C; Jak/STAT signal pathway; immunity; development

Modified publication:

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Discovery of a novel hematopoietin cytokine receptor from teleost fish involved in the Jak/STAT signal pathway

Abstract

Type I cytokine receptors mediate the action of the members of the long chain cytokines canonically involved in numerous physiological function. Here we report a novel cytokine receptor termed Japanese flounder glycoprotein 130 homologue or JfGPH, exhibiting the unique type I cytokine receptor motifs i.e. having a cytokine binding domain (CBD) containing two pairs of conserved cysteine (C) residues, a WSXWS motif, 3 fibronectin domains all in the extracellular region. It is also composed of the Jak binding domains Box 1 and Box 2, and a STAT 3 binding motif (Box 3) in the cytoplasmic region suggesting its mediatory role for Janus kinase/Signal transducers and activators of transcription (Jak/STAT) signal pathway. The JfGPH cDNA is about 3 kb encoding 801 amino acid residues with a predicted molecular weight of 90 kDa and its gene has an 11-exon/10-intron architecture. While JfGPH shows significant homology with the members of type 1 cytokine receptor family including IL6ST (or gp130), IL31α (or GLMR), CSF3R (or GCSFR), LIFR, OSMR, IL12Rβ1 and LEPR, structural and phylogenetic analysis of its protein revealed that it is a novel and an ancestral cytokine receptor found in teleost. JfGPH gene is ubiquitously expressed in Japanese flounder tissues and in a natural embryo (HINAE) cell line showing its critical role in teleost physiological functions similar to gp130 in higher vertebrates. High expression of JfGPH transcripts in immune-related tissues and, in ovary and embryo-derived cell line suggest its role in immune responses, and reproduction/development, respectively. *In vitro* stimulation of spleen, kidney, peripheral blood leukocytes (PBLs) and HINAE revealed that JfGPH is down-regulated by polyinosinic:polycytidylic acid (poly I:C), an interferon (IFN) inducer, suggesting an apparent control of the JfGPH's expression during IFN-induced Jak/STAT signaling.

1. Introduction

Type-1 cytokine receptors are a group of related molecules that mediate the signaling action of class-1 helical cytokines, classified as such based on a shared modular architecture i.e. having a cytokine-binding domain (CBD), fibronectin type-III (FN3) domains, and a signature WSXWS motif (Bazan, 1990; Taga and Kishimoto, 1997). The cytokines these receptors mediate, which include interleukin 6 signal transducer (IL6ST) or glycoprotein 130 (gp130), granulocyte colony-stimulating factor (CSF3), interleukin 6 (IL-6), interelukin 11 (IL-11), ciliary neurotrophic factor (CNTF), leukemia inhibiting factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1) and cardiotrophin-like

cytokine (CLC) likewise share a common tertiary structure i.e. composed of four bundles of α-helices and are involved in numerous physiological processes including immune regulation, host defense, reproduction, development, blood formation and energy metabolism (for review see Huising et al., 2006).

Type 1 cytokines and their cognate receptors form complexes via three interaction epitopes; a site I, located at the distal portion of the D helix (CBD), a site II, composed of residues in the A and C helices that allows for heterodimerization with other receptors and, a site III, a feature only shown in gp130 receptor family located at the N-terminal tip of the D helix- an Ig-like domain (as reviewed by Bravo and Heath, 2000). Upon binding of the cytokine ligand to its receptor, the Janus kinase/Signal transducers and activators of transcription (Jak/STAT) signal pathway is activated. Janus kinases or Jaks are brought to the Box 1 or Jak binding site, a proline rich motif, and in some receptors, to Box 2, a cluster of hydrophobic amino acids followed by positively charged amino acids. Jaks are then subsequently trans-phosphorylated and activated following binding. The activated Jaks then phosphorylate the receptor chain and STATs are recruited through the interaction of the Src homology 2 domain (SH2) with sites of receptor tyrosine phosphorylation such as STAT3 binding motif (Box 3). STATs then form dimers through the intermolecular association of the SH2 domains with the carboxyl sites of tyrosine phosphorylation (Ihle, 1996; Heinrich et al., 1998).

Fish class-1 helical cytokine orthologues have been reported including IL6, IL11, carp cytokine-like (M17), Interleukin 12 (IL12), leptin, erythropoietin (EPO), prolactin (PRL) and growth hormone (GH) (Huising et al., 2006), and recently, the granulocyte colony-stimulating factors (CSF3s) from Japanese flounder, fugu and pufferfish (Santos et al., 2006). Some cognate receptors of these group of signal molecules have likewise been reported in fish particularly from *Tetraodon nigroviridis* genome although their orthology is not yet clear since assignment of names was done only up to the *in silico*-prediction level and the genes were compared only to human (Jaillon et al., 2004). These include Growth Hormone Receptor (GHR), Prolactin Receptor (PRLR), Erythropoietin Receptor (EPOR), Interleukin 12 Receptor γ (IL2Rγ), Interleukin 7 Receptor A (IL7Rα),

Interleukin 12 Receptor β/ Interleukin 4 Receptor A (IL2Rβ/IL4RA), Interleukin 21 Receptor (IL21R), Interleukin 12 p40 (IL12p40), Ciliary Neutrophic Factor (CNTFR), Interleukin 11 Receptor A (IL11Rα), Interleukin 13 Receptor A (IL13Rα), Interleukin 6 Receptor A (IL6Rα), Thrombopoietin Receptor, Interleukin 12 Receptor β2 (IL12Rβ2), glycoprotein 130 (gp130), Leukemia Inhibiting Factor Receptor (LIFR) and Obese Protein Receptor (OBR) or Leptin Receptor (LEPR). Fish cytokine receptors that have been cloned and characterized so far include LIFR and PRLR of gold fish (Hanington and Belosevic, 2005; Tse et al., 2000) and the growth hormones of fugu, zebrafish, Southern catfish and Nile tilapia (Jiao et al., 2006). Information about the kinds of type-1 cytokine receptor molecules in fish and their function in cell signaling e.g. Jak/STAT pathway, which is important in understanding fish physiological processes such as immunity, is very much lacking.

Here, we report a novel cytokine receptor gene that is structurally and phylogenetically related to the class-1 helical cytokine receptors. Because of its close similarity with gp130 compared with other cytokine receptors, we termed it as JfGPH, short for Japanese flounder gp130 homologue. JfGPH appears to be a critical fish receptor because it is ubiquitously expressed in tissues and in an embryo-derived cell line, and is apparently regulated during IFN-induced Jak/STAT signalling.

2. Materials and Methods

2.1. Cell culture

Primary cultures of Japanese flounder peripheral blood leukocytes were prepared using Percoll gradient isolation and placed in Medium B containing RPMI, 5% fetal bovine serum (FBS), and 100 IU ml-1 penicillin G and 100 μg ml-1 streptomycin. Kidney and spleen cells were prepared by slowly mash-filtering tissues using a sterile mesh net and then placed in Medium B. Japanese flounder-derived cell line Hirame Natural Embryo (HINAE) were grown in Leibovitz's L-15 medium (Gibco-BRL, Grand Island, NY)

supplemented with 10% fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS) and 100 IU ml-1 penicillin G and 100 μg ml-1 streptomycin (Gibco-BRL, Grand Island, NY).

2.2. Molecular cloning

The full-length JfGPH cDNA was determined following Santos *et al.* (2006). An Expressed Sequence Tag (EST) clone (Accession no: AU050570) showing putative homology to mouse CSF3 receptor (CSF3R) was used as a probe to screen a Japanese flounder kidney cDNA library. 5' SMART Random Amplification of cDNA Ends (RACE) PCR and PCR cloning was used to complete the cDNA fragment generated from the Japanese flounder cDNA library. On the other hand, the JfGPH gene was completed by "primer walking" using overlapping primers designed from the JfGPH cDNA (Table S1).

2.3. In silico analysis

The nucleotide sequence, translated amino acids, and average molecular weight were analyzed and determined using GENETYX 7.0.3 (GENETYX Corporation). SignalP (http://www.cbs.dtu.dk/services/SignalP/) and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) servers were used to predict signal peptide cleavage and *N*-glycosylation sites, respectively. Identities were calculated using BLASTp (BLOSUM 62) implemented in BLAST 2 SEQUENCES (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) and the complete multiple amino acid alignments were carried out in CLUSTAL X 1.81 using default parameters. The ProDom Server, release 2005.1 (http://protein.toulouse.inra.fr/ prodom/current/html/home.php/) was used to predict protein domains while Phobius (http://phobius.cgb.ki.se/) and TMpred (http://www.ch.embnet.org/ software/TMPRED_form.html) servers were used to identify the transmembrane region.

Table 1. Primers used to complete the Japanese flounder gp130 homologue (JfGPH) gene and for RT-PCR analysis

Primer name	Primer sequence (5' to 3')
F65	CCAGCTGTGGTAACAGGAC
F341	CAGAGAGCTATGCTGCAGTG
F658	CGTATCCGTCAAGTCTGCTA
F39	CTGGGGTCAATTACACGGAGAC
F145	GTTCCAGCCACTGCAGGGAG
F477	GTGCCACTGTGAGCTGGATCA
F1862	TCAAGGAGAAGCCAGTGCCG
F420	GCATGGTTCTGCCAAATCTG
R692	GCAGCGGAGCGTTTCCACTG
R658	TAGCAGACTTGACGGATACG
R413	GTAGGTGCTGATGAAGGTG
R165	GCTCCCTGCAGTGGCTGGAA
R139	CAGAGTCCTCGGCAAACAGA
R39	GTCTCCGTGTAATTGACCCCAG
R165	GCTCCCTGCAGTGGCTGGAAC
R1300	TCTGACTGGCTGAACAGGAGG
R1620	CGTTGGTCCTTCCTGAGTGC
R753	GCTTCCAGAGACACTGAGCAC
R1939	GATGATCTGGTAACAGCCAC
R1295	CTCTGACTCCGATAGGGGCT
R106	CCCTGAGAATGGCCATCACT
R2600	CACAGAGAACCTGGGTGATG

For phylogenetic analysis, we used the unweighted pair group method with arithmetic mean (UPGMA) algorithm implemented in the MEGA3 (http://www.megasoftware.net/index.html/) employing the Poisson method with 1000 bootstrap tests and with complete deletion of gap sites. The bootstrap consensus tree was shown.

2.4. Constitutive expression in tissues and cell lines

Regular RT- and semi-quantitative RT-PCR analysis was carried out following Santos et al. (2006). Primers used for amplification: JfGPH- Forward; 5'-GTGCCACTGTGAGCTGGATCA-3' and Reverse 3'-CTCTGACTCCGATAGGGGCT-5'. For constitutive expression, total RNA was extracted from brain, eyes, gills, kidney, heart, intestine, PBLs, liver, muscle, ovary, skin, spleen, stomach from three (3) apparently healthy Japanese flounder. β-actin was used as a positive control. PCR

conditions were: initial denaturation at 95°C for 5 min, 30 cycles (95°C - 30s, 55°C - 30s, 72°C - 1 min), and final elongation at 72°C for 5 min.

2.5. In vitro stimulation of tissues

Immunostimulation studies was carried out by first extracting total RNA from primary cultures of kidney, spleen and peripheral blood leukocytes (PBLs) from Japanese flounder treated with final concentration of 0.5 mg/ml LPS and polyI:C and sampled at 1, 3 and 6 hrs post-induction. Primers used for amplification: JfGPH- Forward; 5'-GTGCCACTGTGAGCTGGATCA-3' and Reverse 5'-CTCTGACTCCGATAGGGGCT-3'; Mx – Forward; 5'-AACAGCCAAGGCAAAGATTG-3' and Reverse 5`-AATGTCCAGCTCCTCCTTCA-3' (Caipang et al., 2003). β-actin was used as above. PCR conditions were: initial denaturation at 95°C for 5 min, 27 cycles (95°C - 30s, 55°C - 30s, 72°C - 1 min), and final elongation at 72°C for 5 min.

2.6. In vitro stimulation of HINAE cell line

Total RNA was extracted from HINAE cell lines treated with final concentration of 0.5 mg/ml: poly I:C sampled at 1, 3 and 6 hrs post-induction. Primers for JfGPH, Mx and β-actin were the same as above. PCR conditions were: initial denaturation at 95°C for 5 min, 27 cycles (95°C - 30s, 55°C - 30s, 72°C - 1 min), and final elongation at 72°C for 5 min.

3. Results

3.1. Japanese flounder gp130 homologue (JfGPH) gene

Screening of a Japanese flounder cDNA library using an EST fragment corresponding to a CSF3R as a probe yielded a partial cDNA fragment of about 2 kb that includes the polyA tail. Subsequent RACE PCR of the 5' region (confirmed with RT PCR using specific primers) completed the 3 kb cDNA fragment. RT-PCR likewise verified the existence of the full JfGPH transcript (Fig. 2A) BLAST analysis showed that it was most

similar to gp130, thus it was named as Japanese flounder gp130 homologue or JfGPH (Table 1).

JfGPH cDNA is about 3 kb long and has an open reading frame (ORF) of 2,406 bp (Fig.1, Fig. 2B). It encodes for a protein with 801 amino acid residues having a predicted molecular weight of 90 kDa. *In silico* analysis revealed that the JfGPH protein has an extracellular region of 600 amino acids that includes a potential signal peptide of 21 amino acids, followed by a cytokine binding domain (CBD) consisting of two (2) pairs of conserved cysteine residues (aa 117 and 134; 163 and 170), a WSEWS motif, and a sequence with three (3) fibronectin III (FnIII) domains. This region also consists of 19 potential *N*-glycosylation sites. A transmembrane domain consisting of 23 amino acids was predicted at aa 601 to aa 623. The intracellular tail is composed of 178 amino acids with two (2) Jak binding domains, Box 1 (aa 631-636) and Box 2 (aa 696-703), and a Box 3 containing the canonical YXXQ motif (aa 40 – 439) for STAT 3 binding. It also

Table 1. Comparative identity of Japanese flounder gp 130 homologue (JfGPH) with other type 1 cytokine receptor orthologues of different vertebrates.

Gene	Species	JfGPH (%)	Gene	Species	JfGPH (%)
Interleukin 31	*Human*	23	Leukemia Inhibitory	*Human*	20
Receptor A (IL31Rα)	*Mouse*	23	Factor Receptor	*Mouse*	19
or gp130-like	*Chicken*	26	(LIFR)	*Goldfish*	--
monocyte receptor					
(GLMR)			Oncostatin M	*Human*	19
			Receptor (OSMR)	*Mouse*	20
Interleukin 6 Signal	*Human*	21			
Transducer (IL6ST) or	*Mouse*	22	Interleukin 12	*Human*	19
glycoprotein 130	*Chicken*	22	Receptor β1	*Mouse*	--
(gp130)	*Pufferfish*	22	(IL12Rβ1)		
Colony stimulating-	*Human*	23	Interleukin 12	*Human*	--
factor 3 Receptor	*Mouse*	20	Receptor β2	*Mouse*	--
(CSF3R) or	*Frog*	22	(IL12Rβ2)		
Granulocyte colony-	*Rainbow*				
stimulating factor	*Trout*	21	Leptin Receptor	*Human*	--
receptor (GCSFR)			(LEPR)	*Mouse*	--
				Chicken	20
Domeless	*Fly*	--			

-- no significant identity

contains 5 tyrosine (Y) residues (aa 670, 696, 697, 740 and 774), amino acid which is involved in the binding of signaling molecules with SH2 domains (Fig. 2C and 2D). These results indicate that JfGPH is a receptor molecule belonging to the type 1 cytokine receptor family and mediates a Jak/STAT signal cascade.

The JfGPH gene, on the other hand, is about 6 kb consisting of 11 exons and 10 introns (Fig. 1). Exons 1 to 8 encode for the extracellular region, Exon 9 for the transmembrane domain, and Exons 10 and 11 for the cytoplasmic domain that includes the Box 1, 2 and 3 motifs.

Since we supposed that JfGPH is a type 1 cytokine receptor, we next compared its identity with other members of the family (Table.1). As expected with these closely related receptors, JfGPH has significant and about equal identity with known orthologues of IL31Rα, IL6ST, CSF3R and OSMR, the nearest being is the chicken IL31Rα at 26%. Interestingly, however, JfGPH has significant identity with human and mouse LIFR but not with goldfish LIFR, with human but not mouse IL12Rβ1, and with chicken but not with human and mouse LEPR. It has no identity with human and mouse IL12Rβ2 orthologues, and fly Dome even though these receptors are significantly identical with the other receptors of the family. These homologies confirm the assignment of JfGPH to the type 1 cytokine receptor family but at the same time suggest the occurrence of positive selection among the group, and that JfGPH may be a distinct gene.

To know which receptor JfGPH is orthologous to in the absence of a clear homologue, we conducted phylogenetic and structural analysis (Fig. 3). The bootstrap concensus UPGMA tree, which is based on the assumption that the rate of change along the branches of the tree are constant and the distances are approximately ultrameric, showed significant placement of JfGPH outside and at the root of the IL6ST, IL31Rα, CSF3R, IL12Rβ2, LIFR and OSMR clades at 99% bootsrap values suggesting that JfGPH is a unique receptor gene that is novel and appears to be ancestral to some cytokine type 1 receptors. The same sequences were actually run in neighbor-joining (NJ) and maximum parsimony (MP) algorithms (data not shown). Both NJ and MP concensus trees showed

```
TCGACCCACGCGCCGGAGATACAGAGTTAGAAGGGAAACGCGATGTACTGCAGGACACAG   60
TGTGTCTTAACAGCAACGTAAAAACACTGTTATCGAATGCCTCCGGATATCTGACTCCTC  120
TAAATGTGTATTTGAAGGGGAATTTCTTCCAGCTGTGGTAACAGGACAAAATGATGTATC  180
                                                  M  M  Y  P
CCTTCCAAGTGACCTTTATTCTGGCTTTAATCCTCTTCACTTTAAAAGgtgagaratact  240
  F  Q  V  T  F  I  L  A  L  I  L  F  T  L  K  G
tattaacttattaatatcttaatttgtcttgtcggaggtgaggtatgatgtttaaatgca  300
gatgcaggacctgacttgtcaaatgctgctattagagctctgaaaaatgcttcttttgtt  360
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ccgctgcttcaaatggaagtctaagtctgtttaaaatcctcaatcatgtagatttggatg 1020
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ggatgttcagctgcaacatgcaatatcaacattagatatcacaaaattctacacactgta 1440
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catctttctgttagcttggctgaatcatttgcacagatatcttctcatataactcttggc 1560
aagaagacatttatttcacaagatgtcaaagtgtccgttaatgtaacggcttgcatcatt 1620
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                                                           Q
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  N  F  T  Q  Q  R  A  M  L  Q  C  H  S  A  N  T  E  Q  V  L
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  G  G  T  F  I  S  T  Y  T  K  P  S  K  I  S  C  K  L  H  Y
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  C  S  S  H  K  T  R  C  T  C  S  T  N  I  S  G  K  I  Y  L
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  T  S  Y  F  N  V  T  A  R  A  T  T  N  A  W  E  T  Y  S  D
ACCCTCAGGAATTTTATCCCTATTCCATAgtaatttgtcaattatttctatgctcatttc 2280
  P  Q  E  F  Y  P  Y  S  I
tgtatttgaatgtgctgctatgattatgttcccccagTTGGAAATTGTCCCTCCGAAGAT 2340
                                     L  E  I  V  P  P  K  I
AAACGTATCCGTCAAGTCTGCTAATAATGTGTCGATTCAGTGGAGAGCCTCCGCTGCCAG 2400
  N  V  S  V  K  S  A  N  N  V  S  I  K  W  R  A  S  A  A  R
GAGTAGGGAAGACATAAACTGTGAAGTCAAATACACTGAGACTGTCGGTAACAAAACTCC 2460
  S  R  E  D  I  N  C  E  V  K  Y  T  E  T  V  G  N  K  T  P
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                                                 P  I  P
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A  T  C  T  G  T  F  T  Y  A  V  R  Q  I  P  Y  K  Q  H  T
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E  D  E  H  R  I  N  L  R  V  F  N  H  E  T  L  F  A  E  D
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S  V  Y  V  P  A  T  A  G
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                                  S  L  R  H  V  T  E  I  Q  T  S
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 A  V  D  G  V  V  L  V  S  W  T  P  P  V  Q  P  V  R  G  Y
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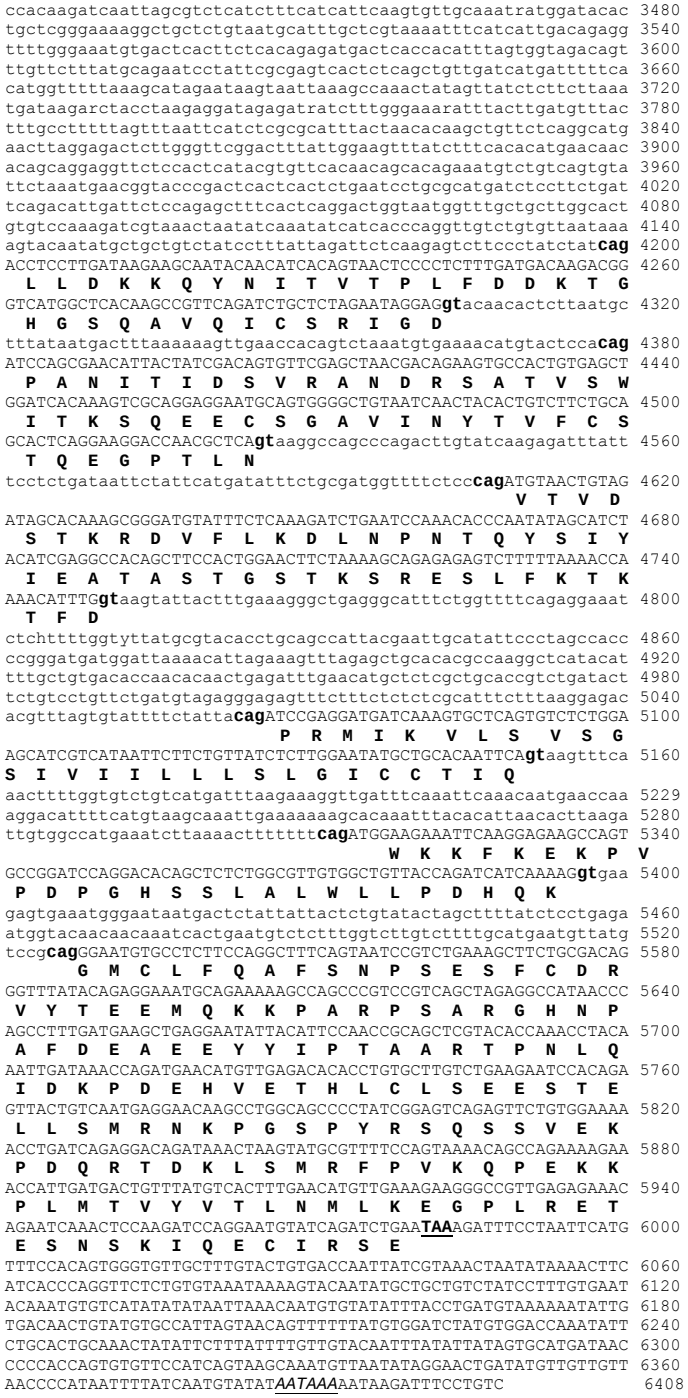

Figure 1. The Japanese flounder gp130 homologue (JfGPH) cDNA and gene (Accession no. AB281273). Exons (*uppercase*), introns (*lowercase*), translated amino acids (*uppercase bold*), start and stop codons (*uppercase bold underlined*), cat-gt junction (*lowercase bold*), polyadenylation signal (*uppercase italics underlined*) are shown.

Fig. 1A

Fig. 1B

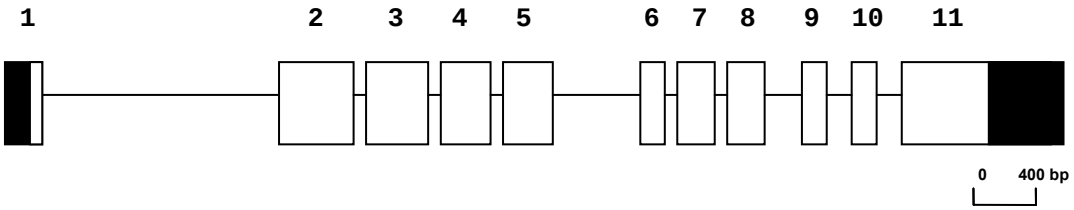

Fig. 1C

```
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KRIDERLSKTINTSLTILSLTNFTQQRAMLQCHSANTEQVLGGTFISTYTKPSKISCKLH  120
YKTLSEGVPQLFTCNWQHKINYSQGINYTVFVSSSSDSMSEMCSSHKTRCTSTNISGKIY  180
LTSYFNVTARATTNAWETYSDPQEFYPYSILEIVPPKINVSVKSANNVSIQWRASAARSR  240
EDINCEVKYTETVGNKTPEVLSHILKPGENGNVSKKLNICTKYNVSVRCALKSAPWSEWS  300
PEKEALTELNKSDVKLHLWRTVTKPDTNGIRKVHAMWMPIPATCDGTFTYAVRQIPYKQH  360
TTGVNYTETLCSNSTCNVEVNEDEHRINLRVFNHETLFAEDSVYVPATAGSLRHVTEIQT  420
SAVDGVVLVSWTPPVQPVRGYMIDWTHDGKQYYWNESKYTNTSLFDLLDKKQYNITVTPL  480
FDDKTGHGSQAVQICSRIGDPANITIDSVRANDRSATVSWITKSQEECSGAVINYTVFCS  540
TQEGPTLNVTVDSTKRDVFLKDLNPNTQYSIYIEATASTGTSKSRESLFKTKTFDPRMIK  600
VLSVSGSIVIILLLSLGICCTIQwkkfkekpvpdpghsslalwllpdhqkgmclfqafsn   660
psesfcdrvyteemqkkparpsarghnpafdeaeeyyiptaartpnlqidkpdehvethl   720
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kegplretesnskiqecirse                                          801
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Fig. 1D

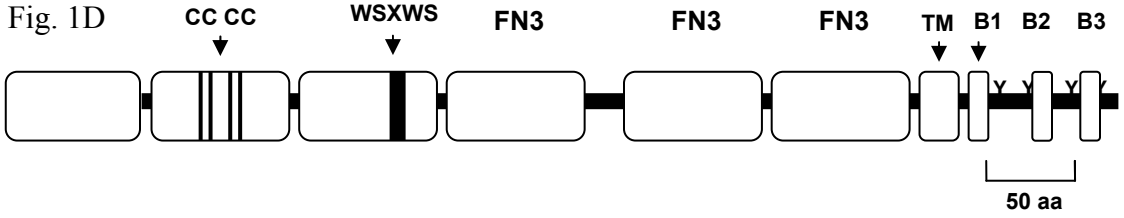


Figure 2. Japanese flounder gp130 homologue (JfGPH) structure and transcript (Accession no. AB281273). A) Gene organization showing 11 exons (*boxes*), 10 introns (*line*) and untranslated regions (*black shade*). B) Amino acid sequence exhibiting the extracellular region (*uppercase*) signal peptide (*uppercase bold*), glycosylation sites (*uppercase italics*), the cytokine binding domain with the four conserved cysteine (C) residues (*arial font bold with connecting line*), WSXWS motif (*uppercase bold boxed*), transmembrane region (*uppercase boxed*), intracellular region (*lowercase*), Box 1 (*lowercase bold boxed*), Box 3 (*lowercase italics boxed*) and tyrosine (Y) residues (*lowercase bold italics*). C) Schematic drawing of the JfGPH protein structure including position of regions in item B plus the 3 fibronectin III (FnIII) domains. D) Partial JfGPH open reading frame (ORF) transcript. Primers F65 and R1295 (Table 1.), which starts from bp 148 and bp 2401, respectively were used to produce the ~2.2 kb amplicon that spans 93 % of the ORF.

that JfGPH is grouping with IL31Rα, but was not conclusive as the bootstrap values supporting the JfGPH node were very low, at only 35% and 17%, respectively. Since human, mouse and chicken IL31Rα and IL6ST are known to tandemly occur, we checked the fugu, pufferfish and zebrafish genomes to see whether such genes and gene arrangement occur in fish and perhaps link JfGPH to a fish "IL31Rα". The fish IL6ST was indeed present (pufferfish – AY374498; fugu - NEWSINFRUG00000140477; zebrafish ENSDARG00000030498, ENSDARG00000053908, ENSDARG00000053957) but a tandem "IL31Rα" was not, nor there was significant BLAST homology between JfGPH and the flanking regions of the fish IL6ST.

We next compared the protein structures of the same receptors (Fig. 4A). JfGPH general protein architecture is very similar to other type 1 cytokine receptors except for the N-terminal region where there is a non-conserved stretch of 95 amino acids starting from the signal cleavage site to the first conserved cysteine (C) residue of the CBD (Fig. 3A and 3B). This fragment, which is suppose to correspond to the Ig-like domain of LIFR, OSMR, IL6ST, CSF3R and IL12Rβ2, does not exhibit any known protein domain as searched through ProDom. In addition, JfGPH clearly exhibits all the binding motifs (Box 1, 2 and 3) similar only to IL6ST and CSF3R. The UPGMA tree result, the absence of a link to a possible fish "IL31Rα", the presence of an N-terminal region with unknown domain, and the cytoplasmic tail motifs lead us to conclude that JfGPH is indeed a novel type 1 cytokine receptor present in Japanese flounder.

3.2. Constitutive expression of JfGPH transcript

Semi quantitative RT-PCR analysis showed that JfGPH is expressed ubiquitously in different tissues/organs (Fig. 5). It is particularly expressed in high amounts in tissues involved in immune responses such as gills, intestine, kidney, blood, liver, skin, spleen, stomach and in reproduction such as ovary. JfGPH is also constitutively expressed in HINAE, whose origin is from embryo cells suggesting further its involvement in reproduction and/or development.

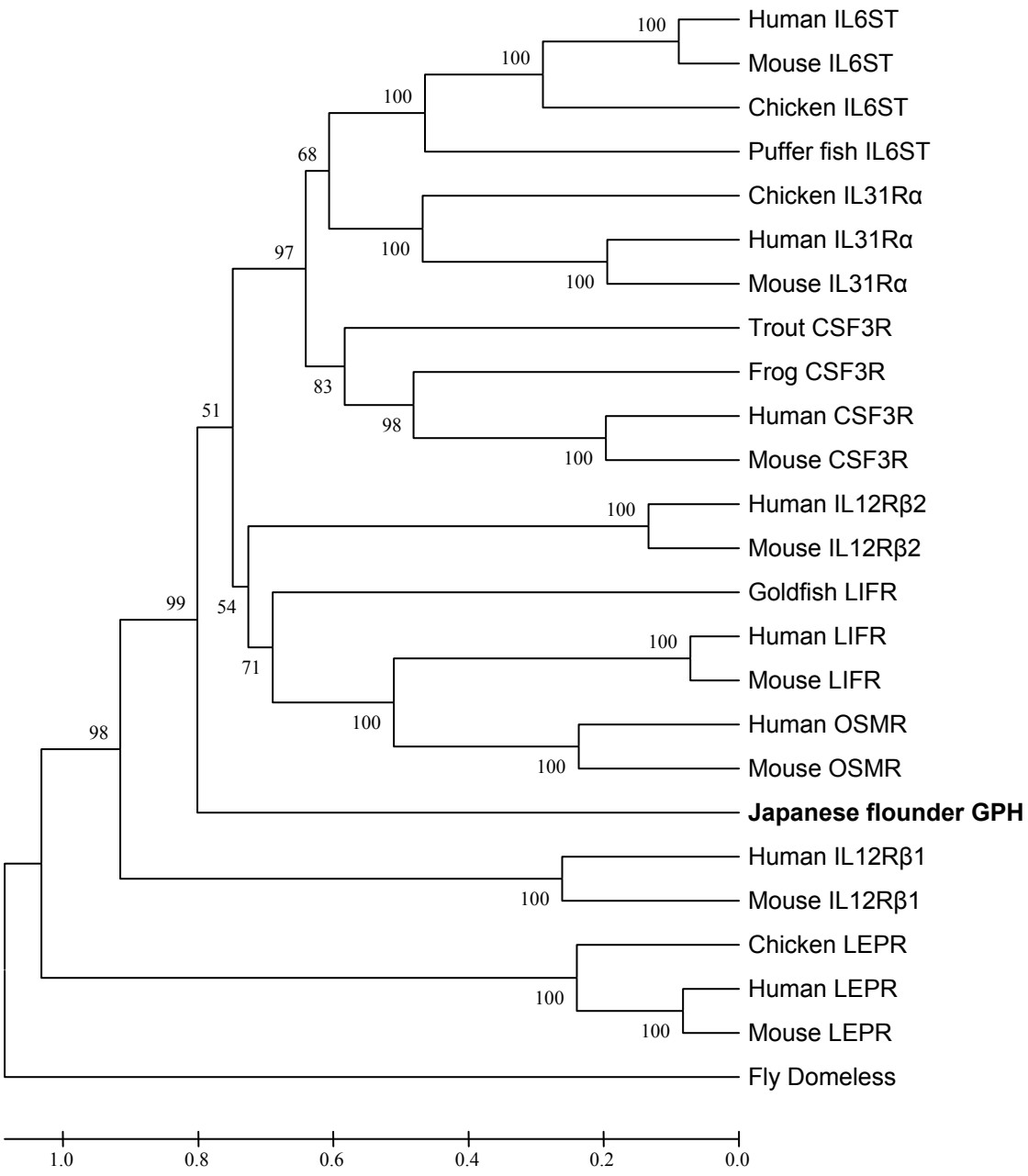
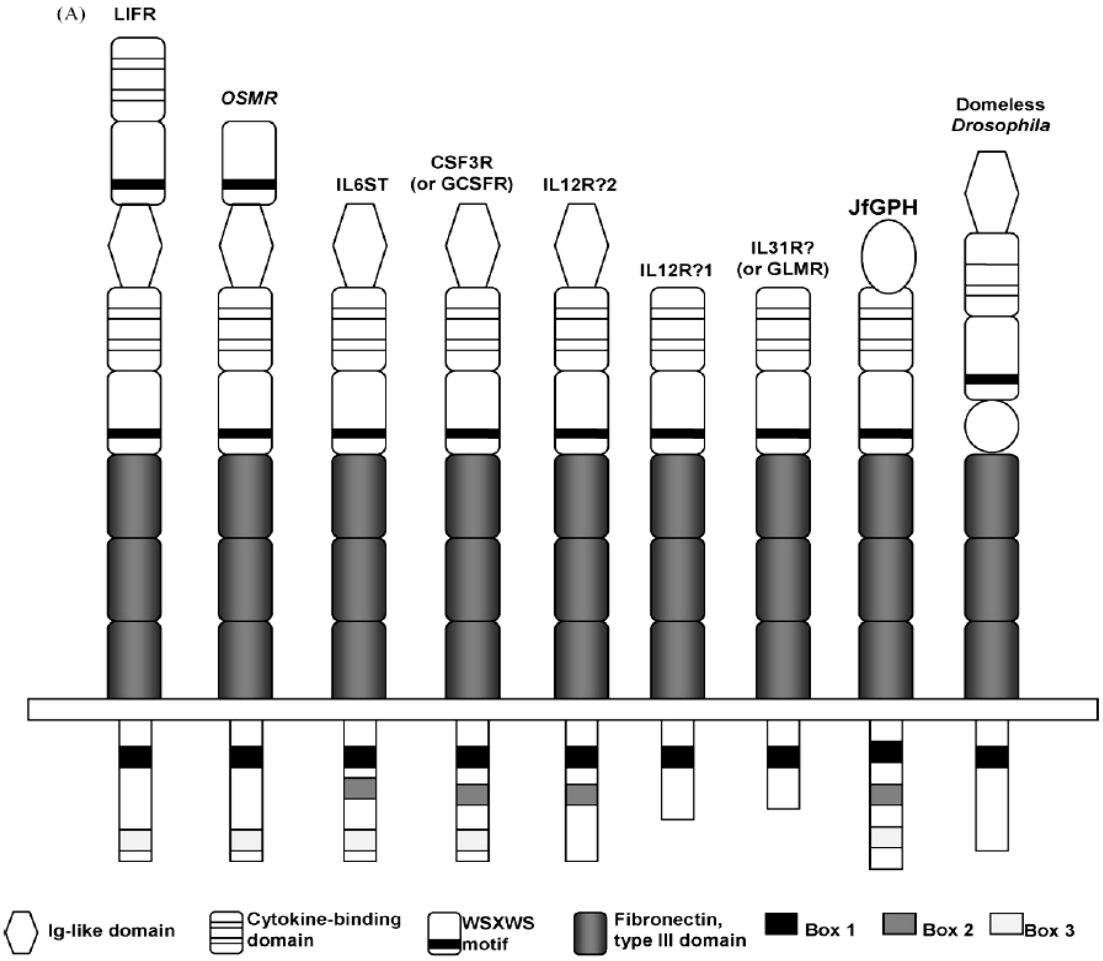


Figure 3. UPGMA tree of type 1 cytokine receptor family including Japanese flounder gp130 homologue (JfGPH). Accession numbers: Human GLM-R (*NP_620586*); Mouse GLM-R (*AF486621*); Chicken GLM-R (*XP_424732*); Human IL6ST (*AAI17405*); Mouse IL6ST (*AAH58679*); Chicken IL6ST (*NP_990202*); Pufferfish IL6ST (*AY374498*); Human CSF3R (*Q99062*); Mouse CSF3R (*AAI16636*); Frog CSF3R (*AAH77792*); Trout CSF3R (*CAE83614*); Human LIFR (*P42702*); Mouse LIFR (*P42703*); Goldfish LIFR (*AAU94362*); Human OSMR (*NP_003990*); Mouse OSMR (*NP_035149*); Human IL12Rβ1 (*P42701*); Mouse IL12Rβ1 (*NP_032379*); Human IL12Rβ2 (*P97378*); Mouse IL12Rβ2 (*NP_032380*) Human LEPR (*AAB09673*); Mouse LEPR (*AAH82551*); Chicken LEPR (*AAF31355*) and Fly Domeless (*AAN64333*).



(B)

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Human IL6ST     ELLDPCGYISPESPVVQLHSNFTAVCVLKEKCMDYFHVNAN--YIVWKTNHFTIPKE--
Mouse IL6ST     Q..E.....Y..F....RG.....I.....A.LQHYY...S--........AAV.R.--
Human CSF3R     -----..H..VSA.I.H.GDPI..S.II.QN.SHLDPEPQ----.L.RLGAE-LQPGGR
Mouse CSF3R     -----..H.EISP...R.GDPVL.S.TISPN.SKLDQQAK----.L.RLQDEP.QPGDR
Human IL31Rα    ------------------------------------------------------------
Mouse IL31Rα    ------------------------------------------------------------
JfGPH           -HMNS.T-VT.KDLYIE.G.DTVVD.--.TS.VSGK--------.F.TL.NKR.DER--
Fly Domeless    --ILDP.WVI.SKVEQLIGGD.NLS.T.N.DYFNGKSAEDCPVEKLYF.GGGRVYRDSK

Human IL6ST     QYTIINRTASSVTFTDIASLNIQLTCNILTFGQLEQNVYGITIISGLPPEKPKNLSC
Mouse IL6ST     .V.V....T.......VVLPSV.......S...I......V.ML..F..D..T..T.
Human CSF3R     .QRLSDG.QE.IITLPHLNHTQAFLSCC.NW.NSL.ILDQVELRA.Y..AI.H....
Mouse CSF3R     .HHLPDG.QE.LITLPHLNYTQAFLFCLVPWEDSV.LLDQAELHA.Y..AS.S....
Human IL31Rα    ----------------------------------------A..-A..E.I..
Mouse IL31Rα    ----------------------------------------VL.T..E.I..
JfGPH           ---LSKTINT.L.ILSLTNFTQ.RAMLQCHSANT..VLG.-.F..TYT--..SKI..
Fly Domeless    HIR.L.N.TILFSD.NAVEQENDYH.MCDEY-----VINKSKVYV.TR.LLVRDFN.
```

Figure 4. Comparison of class 1 helical cytokine receptors with significant identity with Japanese flounder gp130 homologue (JfGPH). A) Schematic drawing of protein motifs and domains (similar to Fig. 2C) are shown. Figure with question mark corresponds to the 93 amino acids in the N-terminal region with no known domain. B) Multiple sequence alignment of the Ig-like domain of IL6ST and CSF3R, and the sequences upstream of the 1st cysteine residue of IL31Rα and JfGPH minus the leader peptide. Conserved amino acids (*dots*), introduced gaps (*dash*) and the 1st conserved cysteine residue of the cytokine binding domain (*boxed*) are shown.

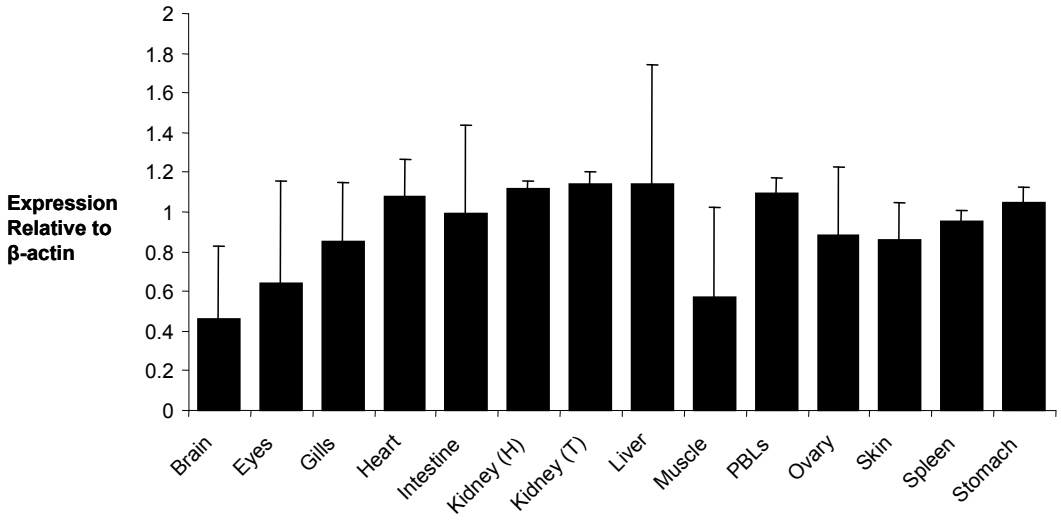

Figure 5. Expression of Japanese flounder gp130 homologue (JfGPH) relative to β-actin in various tissues in Japanese flounder determined by semi-quantitative RT-PCR. Mean values (*bars*) of 3 samples plus standard deviation are shown.

3.3. Regulation of JfGPH expression by interferon-inducing polyI:C

Stimulation of primary cultures of spleen, kidney and peripheral blood leukocytes (PBLs) and of embryonic HINAE cell lines revealed that JfGPH gene expression is down-regulated by polyI:C in a time-series manner *in vitro* (Fig. 6). Correct stimulation by polyI:C was confirmed by the up-regulation of Mx while the integrity of the RNA extraction and cDNA synthesis was verified through the expression of β-actin.

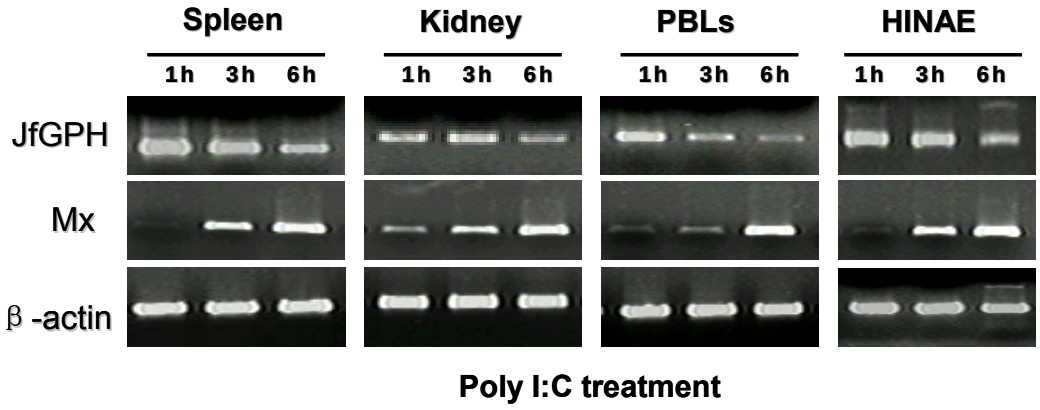

Figure 5. Expression of Japanese flounder gp130 homologue (JfGPH) gene in response to polyinosinic:polycytidylic acid (poly I:C). JfGPH, Mx and β-actin transcript expression *in vitro* in Japanese flounder spleen, kidney, PBLs and HINAE at 1, 3 and 6 hrs post-stimulation with polyI:C.

4. Discussion

Here we report a novel molecule that belongs to the type 1 cytokine receptor family found in fish. It exhibits the classic structural features of the family i.e. having a cytokine binding domain with the four (4) conserved cysteine residues, a WSEWS motif, three FNIII domains in its extracellular region, and 2 Janus kinase (Jak) binding motifs (Box 1 and Box 2), and a STAT3 binding domain (Box 3) in the intracellular tail. Subsequent structural and evolutionary analysis of the protein revealed that JfGPH is another long type 1 cytokine receptor, which does not exhibit an Ig-like domain in its N-terminal region unlike IL6ST and CSF3R, but instead possess a stretch of amino acids with no known domain or function. Such discovery is not surprising as a cytokine type-1 receptor in human has only been cloned and characterized just recently (Ghilardi et al., 2002; Diveu et al., 2003 and Dreuw et al., 2004).

JfGPH is clearly involved in a Jak/STAT signal transduction pathway because of the conserved Box 1, Box 2-like and Box 3 motifs, and tyrosine (Y) residues in its cytoplasmic tail. Box 1 is a membrane-proximal, proline rich motif which could associate the Janus kinase (Jak) family upon ligand binding while the hydrophobic Box 2 could also serve as a docking site for Jaks. Box 3 is established to be the binding site of STAT3 signal molecules. While it may be expected that fish do involve Jak/STAT signaling, JfGPH structure together with the identification of Japanese flounder Jak2 (EST accession no. AU091091), STAT1 (EST accession no. AU261169) and STAT3 (EST accession no. AU083083) ESTs (Table 2) reinforces this idea. The Jak/STAT pathway is responsible for numerous physiological responses including hematopoieis, immune responses and development in mammals (Hou et al., 2002; Ihle, 1996) and even in development and antiviral responses in drosophila (Chen et. al., 2001; Agaisse and Perrimon, 2004; Costert et al., 2005). With the presence of the CBD and three (3) FN3 domains in the extracellular region of JfGPH, it is likely that it utilizes 2 (site I and site II) of the 3 binding sites used by the long chain cytokines to bind to their receptors (Bravo and Heath, 2003). Hence, we speculate that a specific ligand for JfGPH binds to the receptor's binding site I or II or perhaps III and allows for the binding of JfJak to its Box

1 or Box 2. This in turn signals the recruitment of the JfSTAT3 in the Box 3 and undergoes phosphorylation. The phosphorylated STAT3s form dimers and translocates to the nucleus to effect target gene expression. We show a diagram of such putative signal cascade mediated by the JfGPH in Fig. 6. The ligand for JfGPH is not yet known and it is not clear whether JfGPH utilizes the peptide sequence that is parallel but not similar to the Ig-like domain for a site III interaction or whether JfGPH functions as a homodimer or a heterodimer similar to gp130. These unknowns warrant further study to better understand JfGPH function.

Table 2. Identities of putative Japanese flounder Jak, STAT1 and STAT3 with known orthologues from other species.

Japanese flounder (EST accession no.)	**Zebrafish**	**Human**
AU091091	62% (Jak2)	61% (Jak2)
AU261169	51% (STAT1)	37% (STAT1)
AU083083	83% (STAT3)	76% (STAT3)

Interestingly, while JfGPH shows significant homology with all known IL31Rα, IL6ST, CSF3R, and OSMR genes, it does not have identity with every LIFR, IL12Rβ1, IL12Rβ2 and LEPR orthologues, in particular the goldfish LIFR, suggesting that JfGPH and perhaps the type 1 cytokine receptor family in lower vertebrates is undergoing a certain level of positive selection, which is not an unusual event for cytokine molecules (Huising et al., 2006; Jaillon, et al., 2004, Santos et al., 2006). It is believed that, tandem gene duplication potentially expanded the type 1 cytokine and receptor genes in vertebrates as evidenced by their short physical distances. In addition, short type 1 cytokine and receptor genes may have occurred later in evolution than long type I cytokines and receptors accounting for the more developed acquired immunity in higher vertebrates (Boulay et al., 2003). The identification of a novel long type 1 cytokine receptor suggests that this group is also expanding at least in teleosts. Just recently, we have cloned a partial cDNA fragment which surprisingly appears to have identities with chicken IL12Rβ, chicken OSMR, frog gp130 and frog PRLR (unpublished data). This variation could also be the reason why our BLAST search of existing fish genomic databases through *Ensembl* could not identify a homologous JfGPH gene.

The phylogenetic tree in this study resembles the tree generated for the group 2: gp130 family by Boulay et al. (2003), which also suggested that this same group likely represents the ancestral forms of the type-1 cytokine receptors. It is interesting to note that JfGPH appears to be a molecule ancestral for the type-1 cytokine receptors because of its cross similarity and basal phylogeny. However, it has no significant homology with the drosophila Domeless receptor, which is said to be the ancestral molecule for the mammalian cytokine receptor family (Chen et al., 2001). It is possible that JfGPH has ignificantly diverged from the Domeless receptor due to selection as mentioned previously.

High expression of specific JfGPH transcripts in kidney, spleen, kidney, PBLs liver, skin, stomach and gills suggest that it is involved in immune responses. Moreover, JfGPH's significant expression in ovary and in a cell line whose origin is from embryo cells shows that it has a role in reproduction and development. This highlights the important role of JfGPH in the physiological processes in fish similar to gp130, a type 1 cytokine receptor well studied in higher vertebrate. Gp130 has been established to be a critical receptor molecule to an organism such that mutation or knockdown of the said gene has been fatal to the organism (Kishimoto et al., 1995).

The involvement of JfGPH in immunity and particularly in the Jak/STAT pathway is further confirmed through the down-regulation of its expression following polyI:C stimulation in tissues and in cell lines. polyI:C is a double stranded RNA known to induce IFN-α/β production. We speculate that the IFN-induced Jak/STAT pathway somehow inhibits the expression and thus the function of JfGPH-dependent Jak/STAT signaling presumably to regulate and balance the system. Actual mechanism though should be confirmed and fully explored. Down regulation of JfGPH by polyI:C is in contrast to the up-regulation of GPL, IL12Rβ2 and IL23R β1, receptors homologous to JfGPH, by IFN-γ treatment in monocytes and dendritic cells (Diveu et al., 2003; Parham et al., 2002). The difference in JfGPH expression as compared with other receptors further reflects its uniqueness at the transcriptional level.

It is important to identify the ligand(s) that could specifically bind to JfGPH and confirm the Jak/STAT signal cascade it would induce. Four (4) cytokines of the IL-6 cytokines from Japanese flounder namely *po*CSF3 (Santos et al., 2006), IL6 (Nam et al., 2006), IL11 type b (unpublished data) and an M17 homologue (submitted) have been cloned and are potential ligands. Identification of the JfGPH ligand could greatly help in understanding further the biological function of JfGPH and other related receptors.

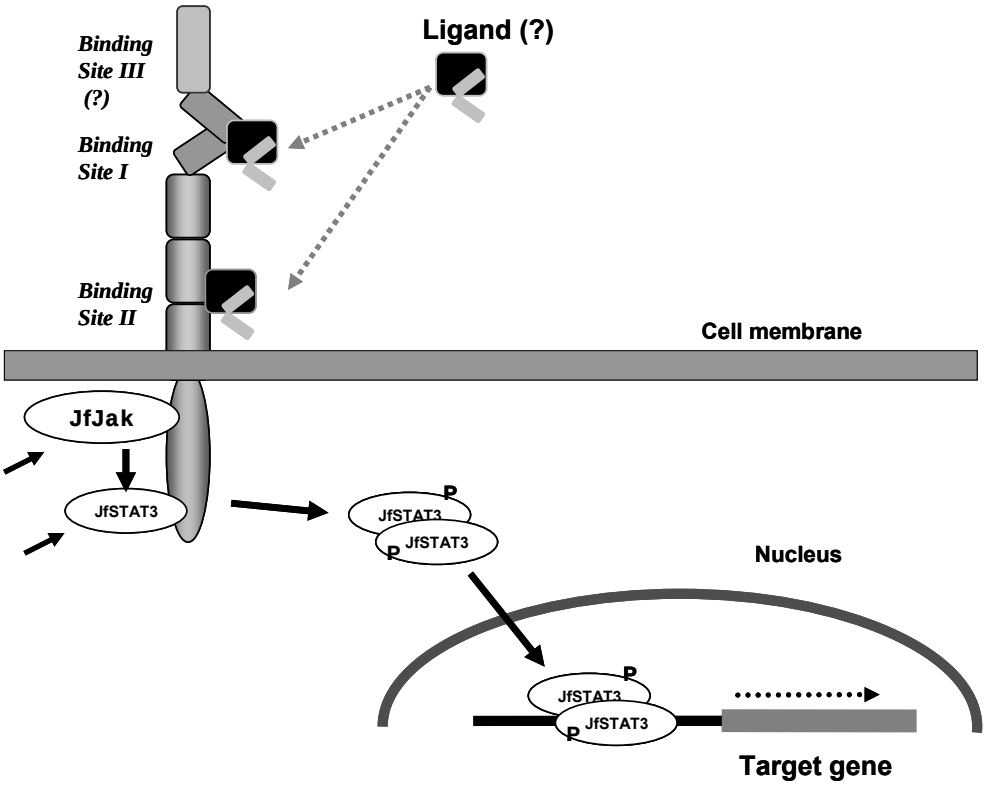

Figure 7. Diagram of the putative Janus kinase/ Signal transducers and activators of transcription (Jak/STAT) signal cascade in Japanese flounder mediated by Japanese flounder gp130 homologue (JfGPH). Japanese flounder Janus kinase (JfJak), Japanese flounder signal transducers and activators of transcription 3 (JfSTAT3) and ligand binding sites are shown. Specific ligand for JfGPH and Site III's putative function as a cytokine binding site are unclear (?).

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CHAPTER 5

Molecular tools for studying immuno-hematopoiesis in Japanese flounder, *Paralichthys olivaceus*: recombinant protein production and polyclonal antibody-based cell surface markers

5.1. Production of a recombinant Japanese flounder CSF3 protein in a fish cell line, Hirame Natural Embryo (HINAE)

Keywords: Japanese flounder (*Paralichthys olivaceus*); granulocyte colony-stimulating factor, recombinant protein, Hirame natural embryo (HINAE)

Modified publication:

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Production of a recombinant Japanese flounder CSF3 protein in a fish cell line, Hirame Natural Embryo (HINAE)

Abstract

Granulocyte colony-stimulating factor (CSF3), a cytokine involved in neutrophil development, has been successfully utilized as a recombinant protein drug for cancer therapy in humans and domestic animals. The Japanese flounder CSF3 has been identified previously. Here, we succeeded in producing and 26-kDa recombinant CSF3 protein in a fish cell line (Hrame Natural Embryo or HINAE) using a mammalian expression vector pCDNA4 HisMax C. Its expression was found to increase with time. The recombinant CSF3 fusion protein may be useful in further functional studies or applications.

1. Introduction

The granulocyte colony-stimulating factor (CSF3) is a 4 α-helical long chain cytokine involved in development, maturation and survival of the neutrophilic lineage (as reviewed by Basu et al. 2002, Barreda et al., 2004) ad is one of the successful cytokine therapeutics in humans and domestic mammals, which include interferons and hematopoietic growth factors (colony stimulating factors). Recombinant CSF3 protein is commercially available under the generic name filgrastim (brand name: Neupogen) or pegfilgrastim (brand name: Neulasta). These therapeutics are used for supporting cancer patients receiving chemotherapy or bone marrow transplants, patients under peripheral-blood-progenitor-cell collection and therapy, and patients with neutropenia (Welte et al., 1996; Vilcek and Feldmann, 2004). Teleost fish has been successfully identified and characterized in Japanese flounder, *Paralichthys olivaceus*, fugu, *Takifugu ruripes* and in green spotted pufferfish, *Tetraodon nigroviridis* (Santos et al., 2006) Recombinant teleost CSF3, however, has not been produced and studied for its function and potential therapeutic application.

2. Materials and Methods

2.1. Plasmid construct

A DNA fragment containing the full CSF3 ORF (containing 633 bp and a predicted mass of 21 kDa) and an added *Bam*H1 and an *Eco*R1 site was generated by PCR using designed primers *po*CSF3-FProt and *po*CSF3-RProt (Table 1). The mammalian expression vector pCDNA4/HisMax C and the PCR fragment were cut using *Bam*H1 and *Eco*R1 restriction enzymes, recovered using EASYTRAP ver. 2 (Takara, Japan) and ligated together using ligation high (TOYOBO, Japan). The resulting expression vector construct was termed here as pCDNA4-CSF3 construct (Fig. 1).

Table 1. Primers used in this study

Species	Primer name	Primer sequence
Japanese flounder (*Paralichthys olivaceus*)	poCSF3-F2	ATGGACTCTGAGACAGTTGT
	poCSF3-R	CGGTAACTGCTTAGCGTGCA
	poCSF3-FProt	TTTGGATCCATGGACTCTGAGACAGTTGT
	poCSF3-RProt	TTTGATTCTTAGCGTGCACCTGCAGCTCGGC

2.1. Transfection and detection

HINAE cells seeded onto 35-mm tissue culture plates were transiently transfected with the pCDNA-HisMax-CSF3 construct using Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's protocol. Two (2) each of 6 plates were incubated for 1, 2 and 3 days with pCDNA4-CSF3 and 2 plates were mock infected with blank pCDNA-HisMax C.

Transfected cells were harvested accordingly using a rubber scraper and then pelleted by centrifugation at 1,500 x g for 5 minutes. The 6 plates were separated into 2 sets, one for RT-PCR and the other for western blot. For RT-PCR analysis, the above method was used. For the recombinant protein, harvested cells were resuspended in 20 µl Phosphate Buffered Saline (PBS). This was then homogenized using a cell lysis buffer (50 mM Tris-HCl, pH 7.8; 150 mM NaCl; 1% Nonidet P-40). Samples were diluted with equal amounts of SDS sample buffer (125 nM Tris HCl, pH6.8; 4 % SDS; 10 % glycerol; 10 % 2-mercaptoethanol; 0.004 % bromphenol blue) and boiled for 10 minutes. The samples

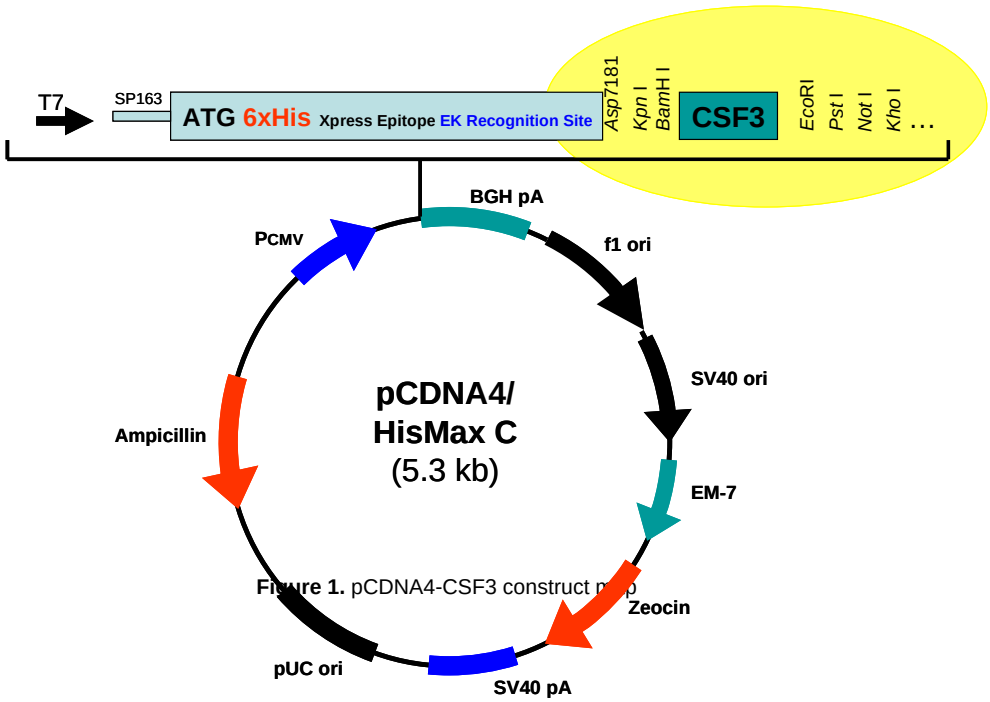

Figure 1. pCDNA4-CSF3 construct map

were resolved in 15 % SDS PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was then incubated with AntiXpress antibody (Invitrogen, USA) at a 1:10,000 dilution. Recombinant CSf3 protein was then detected by using anti-mouse IgG conjugated with alkaline phosphatase (Promega, USA) following the manufacturer's protocol.

3. Results

Three clones of the pCDNA4-CSF3 construct (C6, C18, and C27) were confirmed to be in frame by sequencing (Fig. 1). These same clones were transfected to HINAE cells and were able to produce the recombinant mRNA transcript of about 700 bp in size as shown by RT-PCR analysis (Fig 2.A). pCDNA4-CSF3 construct C6 was selected and transfected again to HINAE cells for protein expression. Western blot analysis detected a 26-kDa protein (Fig 2.B) and showed that its amount increased at the 3rd day of incubation. We tried to produce recombinant CSF3 with bacterial expression systems including pQE, pET32, pET28 and pNCMO2 for *Bacillus brevis* but with negative results.

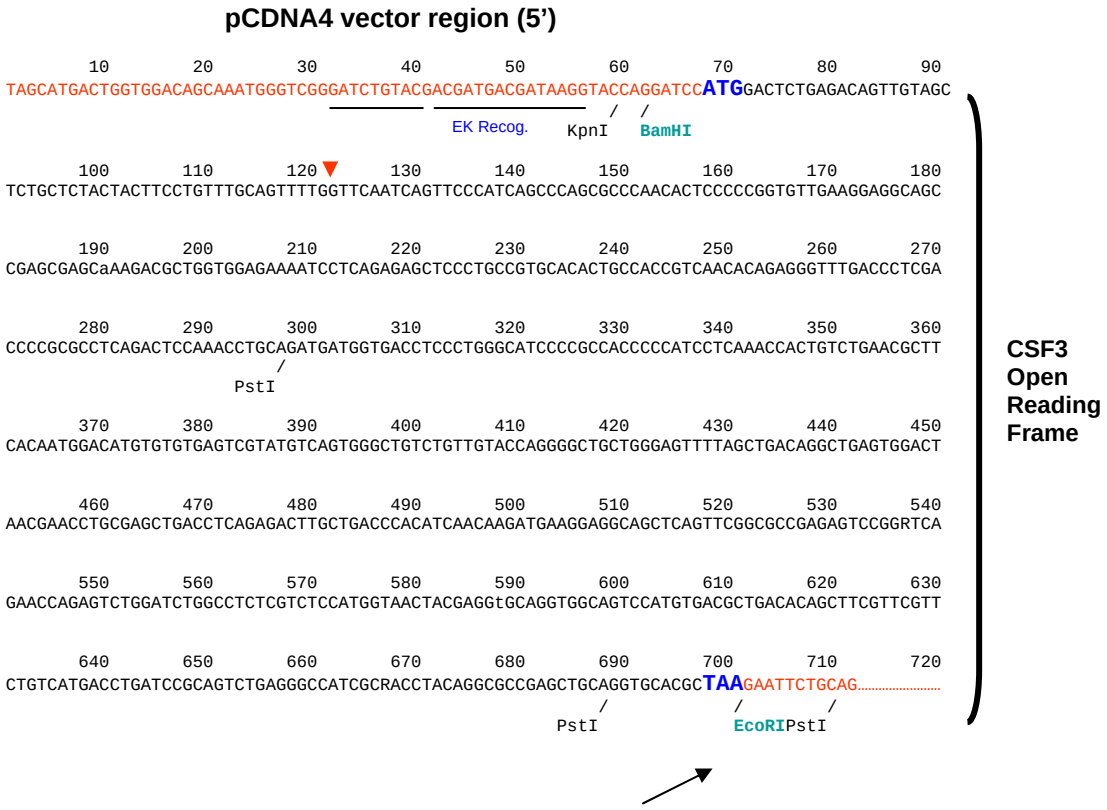

Figure 2. Confirmed sequence of the pCDNA4-CSF3 construct map

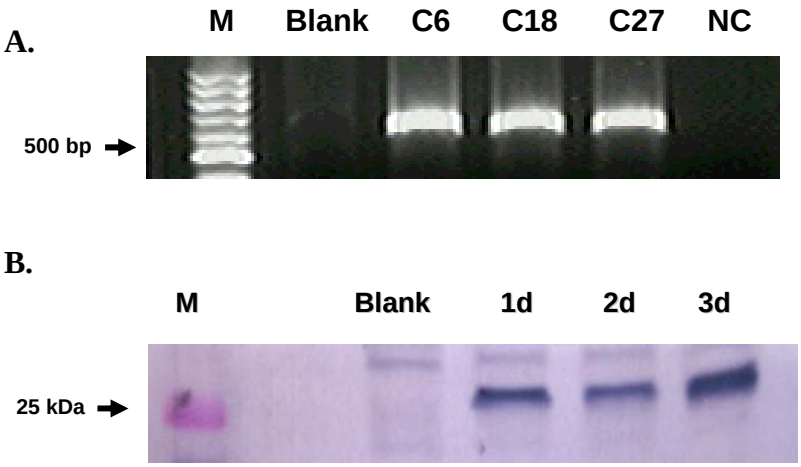

Figure 3. Recombinant CSF3 production using the mammalian expression vector pCDNA HisMax expressed in HINAE cells: A) CSF3 mRNA transient expression shown through RT-PCR; M-100 bp ladder, Blank-pCDNA Hismax C, C6 - pCDNA Hismax C + CSF3 ORF (clone 6), C18 - pCDNA Hismax C + CSF3 ORF (clone 18), C27 - pCDNA Hismax C + CSF3 ORF (clone 27), NC – PCR negative control; B) Recombinant CSF3 expression detected using AntiExpress Antibody in Western blot. M- protein marker, Blank – pCDNA Hismax C, 1d – recombinant CSF3 (day 1), 2d – recombinant CSF3 (day 2), 3d – recombinant CSF3 (day 3).

4. Discussion

We were successful in constructing a mammalian expression vector producing the recombinant CSF3 protein of Japanese flounder in a fish cell line *in vitro*. The implication of such is that the recombinant protein produced is fully functional. Why bacterial expression systems failed to produce recombinant CSF3 is unclear. The immune-related activity of Japanese flounder CSF3 against possible pathogens, together with an understanding of its regulatory regions, suggests that the recombinant CSF3 protein is a good candidate for enhancing immune responses against disease. For example in mice that have been vaccinated with HIV-1 *env* and *gag/pol*, co- delivery of recombinant mice CSF3 with macrophage colony-stimulating factor (MCSF) resulted in a moderate effect on serum antibody responses and T-helper cell proliferation, upregulated INF-γ production in antigen-stimulated splenocytes and increased the serum IgG_2/IgG_1 antibody isotype ratio (Kim et al., 1999).

Acknowledgements

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CHAPTER 5

Molecular tools for studying immuno-hematopoiesis in Japanese flounder, *Paralichthys olivaceus*: recombinant protein production and polyclonal antibody-based cell surface markers

5.2. Effective polyclonal Japanese flounder IgM antibodies derived from recombinant Japanese flounder IgM protein fragments

Keywords: Japanese flounder (*Paralichthys olivaceus*); type-1 cytokine receptor; novel; poly I:C; Jak/STAT signal pathway; immunity; development

Modified publication:

Santos M.D., Saito-Taki, T., Kondo H., Hirono I., Aoki T., 2007. Effective polyclonal fish IgM antibodies derived from recombinant fish IgM protein fragments. *Prepared for Journal of Immunological Methods*

Effective polyclonal Japanese flounder IgM antibodies derived from recombinant Japanese flounder IgM protein fragments

Abstract

Immunoglobulins (Igs) are parts of the humoral immune response secreted to neutralize specific antigens. They are used to monitor and evaluate immuno-competence and disease resistance in animals. To do this, polyclonal (Pab) and monoclonal (Mab) antibodies against Igs are produced to measure Ig levels. Pabs are easy and relatively inexpensive to produce but are usually less stringent, while Mabs are more difficult and costly to develop but are usually sensitive and specific. In Japanese flounder, *Paralichthys olivaceus* (a commercially important aquaculture species) Mab against IgM derived from purified serum IgMs have been developed and tested. Here, we developed polyclonal mouse anti- Japanese flounder IgM using recombinant IgM protein fragments expressed by the pET bacterial expression vector translated in *Escherichia coli*, BL21+. The 2 IgM protein fragments correspond to parts of the IgM conserved region, the N-terminal part named IgM fragment 1 (IgM1) is ~23 kDa and the C-terminal part named IgM fragment 2 (IgM2) is ~12 kDa. These proteins were 6X His-tag purified and then used as antigens for antibody production. Western blots showed that the 2 polyclonal antibodies (Pab-IgM1 and Pab-IgM2) could sensitively detect the ~77 kDa heavy (H) chain in denatured conditions in Japanese flounder tissues. Only Pab-IgM1 however, was able to detect the second 72 kDa H chain and is specific to Japanese flounder. IgM protein expression was shown to be relatively higher in immune-related tissues and that serum IgM under denaturing conditions showed possible redox forms. Immunohistochemistry analyses showed that the Pabs-IgMs could detect IgM+ plasma cells *in vivo*. Taken together, we have developed useful Japanese flounder IgM Pabs, particularly the Pab-IgM1, that are comparable to reported IgM Mabs in terms of specificity and sensitivity, but is relatively less time consuming and is more cost-effective.

1. Introduction

Immunoglobulins are part of the humoral immune system in fish secreted to neutralize antigens in a specific manner also induces the activation of the complement cascade (Rijkers, 1982). Two Igs have been described in teleost fish, IgM and IgD, since the early late 90's (Pilstrom and Bengten, 1996; Wilson et al., 1997). Recently, however, other forms of fish immunoglobulins have been identified including IgT in rainbow trout, *Oncorhynchus mykiss* (Hansen et al., 2005) and IgZ in zebrafish, *Danio rerio* (Danilova et al., 2005).

Teleost IgM exists as membrane bound or secreted forms consisting of 2 subunits, the heavy chain (μ) and the Light chain (L) at ~ 70kDa and ~25 kDa, respectively. Teleost fish IgMs shows diversity in secreted "redox" forms (Kaattari et al., 1988). This has been thought to be as a result of differing disulfide bonding between adjacent heavy chains plus the presence of non-covalent bonding, which is being used by teleost fish to generate antibody diversity.

IgMs are being used to evaluate immunocompetence and disease resistance in commercially important species using monoclonal antibodies directed against these IgMs. Japanese flounder, *Paralichthys olivaceus*, is an important mariculture fish species and monoclonal antibodies (Mabs) against its IgM have been developed from purified serum IgM (Jang *et al*., 2004). Another commercially important species and a close relative to Japanese flounder, the Atlantic halibut, *Hippoglossus hippoglossus*, has also Mab developed against its IgM (Grove *et al.,* 2006). Using these Mabs, it was observed that Japanese flounder serum Igs had 2 heavy chains at 72 kDa and 77 kDA, and 2 Light chains at 26 kDa and 28 kDa. On the other hand, Atlantic halibut serum IgM was composed of 1 heavy chain at ~ 76 kDa and 6 Light chain variants at ~25 to ~28.5 kDa.

While, Mabs are more specific and sensitive, polyclonal antibodies are cost-effective and are also useful for certain assays and depending on the resulting sensitivity of the Pab produced. Previously, we have shown that Pabs derived from recombinant proteins for *in situ* assays of fish immune-related gene function in Japanese flounder have been successful (Takano et al., 2007; Takano et al., 2006; Lin et al, 2005). Here, we report the production of 2 mouse polyclonal anti-Japanese flounder IgM antibodies (Pab-IgM1 and Pab-IgM2) derived from recombinant Japanese flounder IgM protein fragments derived from the IgM conserved constant region. These Pabs were shown to be sensitive to detect IgM in denatured form as well as *in vivo*, and is specific to Japanese flounder in the case of Pab-IgM2. These results therefore suggest that the Pabs were useful and cost-effective tools to further the study of Japanese flounder immune responses and system.

2. Materials and Methods

2.1. Plasmid construction

Two DNA fragments containing the IgM conserved region (Fig. 1) with added *Nde*I and an *Eco*R1 sites were generated by PCR using designed primers (Table 1). The bacterial expression vector pET 32 (Fig. 2) and the PCR fragment were cut using *Nde*I and *Eco*R1 restriction enzymes, recovered using EASYTRAP ver. 2 (Takara, Japan) and ligated together using ligation high at 16^{o}C overnight (TOYOBO, Japan). These were then transformed to competent *Escherichia coli* JM109 cells using the heat-shock method and cultured overnight at 37^{o}C on Luria-Bertani (LB) agar containing ampicillin (AP) (100 ug/ml). Resulting bacterial colonies were then cultured in LB-AP overnight for plasmid extraction and purification using the alkaline-lysis method. Confirmation of positive expression vector constructs was done by automated sequencing using the ThermoSequenase kit (Amersham Biosciences, Piscataway, NJ) on a LC4200 automated DNA sequencer (Li-Cor, Lincoln, NE). Confirmed pET32-IgM1 and pET-IgM2 constructs were each transformed to *E. coli* BL21 codon + using the heat-shock method and culture-selected in LB-agar plates containing Amp (100 ug/ml) and chloramphenicol (CP) (30 ug/ml) overnight. Resulting bacterial colonies were then used for protein expression and purification.

2.2. Protein expression and purification

To check and select clones expressing well IgM protein fragments, an initial small-scale experiment was performed. Selected clones were cultured overnight at 3 ml LB-AP/CP at 37^{o}C. From these, 200 ul was taken and inoculated in fresh 2 ml LB-AP/CP broth at 37^{o}C until OD_{600} of about 0.6. These were then induced with 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hrs. An empty pET32 vector and non-induced cells were used as controls. Resulting cultures were centrifuged and the cell pellets were resuspended in 300 ul phosphate buffered saline (PBS). From this suspension, 20 ul was mixed with 2X SDS buffer and boiled for 10 min. After which 20 ul were run in a 15% SDS-PAGE for about 1.5 hrs. at 30 milli Amperes. Coomassie brilliant blue staining and Western blotting using an anti-His antibody (Promega, Madison, WI) were used to visualize expressed protein.

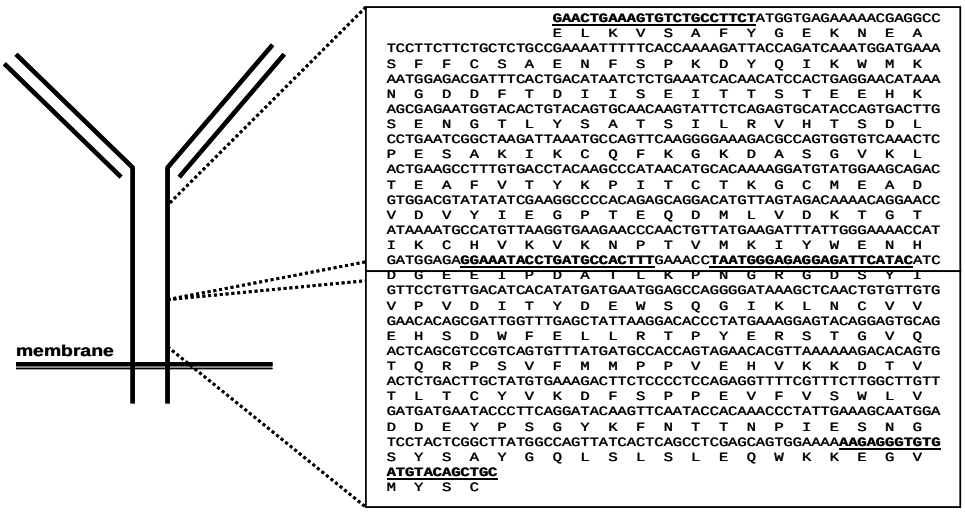

Figure 1. Sequence and location of the IgM constant region fragments that were used to generate the recombinant proteins.

Table 1. Primers used in this study

Species	Primer name	Primer sequence
Japanese flounder (*Paralichthys olivaceus*)	IgM1F	TTTTCATATGGAACTGAAAGTGTCTGCCTTCT
	IgM1R	TTTTGAATTCTTAATGATGGTGATGATGGTGTGATGTCAACAGGAACGATG
	IgM2F	TTTTCATATGGAATGGAGCCAGGGGGATAAAG
	IgM2R	TTTTGAATTCTTAATGATGGTGATGATGGTGGCAGCTGTACATCACACCCT

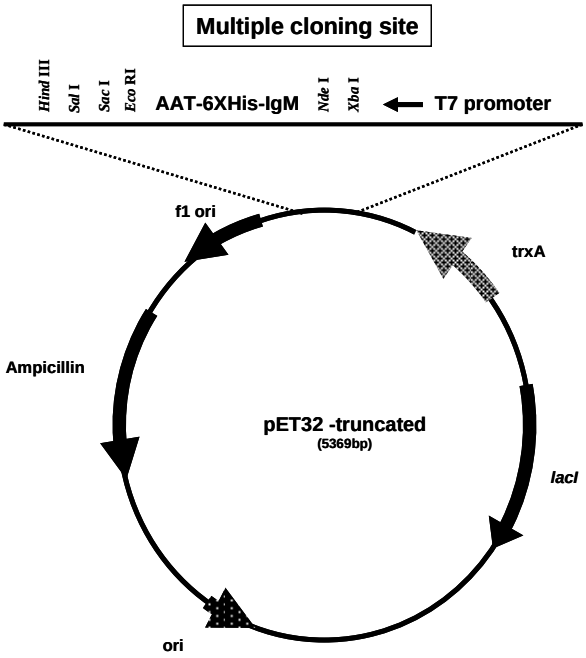

Figure 2. Bacterial expression system pET32-truncated used to produce the recombinant Japanese flounder IgM1 and IgM2 protein fragments.

Large-scale expression of Japanese flounder IgM1 and IgM2 was carried out. Briefly, a 2 ml overnight LB-AP/CP culture of selected clones was placed in 300 ml LB-AP/CP and then cultured again overnight at 37^{o}C. Cultures were centrifuged and then resuspended in PBS. After which, freeze-thaw method and then sonication (20 sec at 20 amplitude) were performed to lyse the cells. Sonicated samples were centrifuged, and the pellet resuspended in PBS, then filtered in 0.45 uM filter (Millipore). Recombinant IgM1 proteins were purified from inclusion bodies by metal affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) agarose beads (Qiagen K.K Tokyo, Japan). Briefly, inclusion bodies were solubilized in denaturing buffer (8.0 M Urea, 100 nM NaH_2PO4, 10 mM Tris-HCL, 20 mM imidazole, pH 8.2) for 12 hrs and centrifuged at 6,000 rpm for 30 min at 10oC. Supernatant was applied to the Ni-NTA beads in propylene column, which were resuspended in wash buffer (50 mM Na_2HaPO4, 300 mM NaCl, pH 8.2). Washing was conducted with was buffer containing 30 mM and 100 mM imidazole. Elution was performed with wash buffer containing 250 mM imidazole. Eluents were then subjected to filter sterilization using 0.22 dialysis um Millex^{TM} Syringe Driven Filter Unit (Millipore) and then dialyzed overnight in 3L 1X PBS. Purified recombinant proteins were visualized by SDS-PAGE and CBB staining and quantified using the bicinchoninic acid method in the BCA^{TM} Protein assay kit (Pierce, Rockford, Il, USA).

2.3. Polyclonal antiserum production

Purified IgM1 (738 ug/ml) and IgM2 (2250 ug/ml) were intraperitoneally injected (IP) into each of 5 BALB/c strain. IPs were administered at 2-weeks intervals at the following volumes; 0.1 ml, 0.15 ml, 0.2 ml and 0.2 ml. The mice were bled and the pooled serum was designated as IgM1 Pab and IgM1 Pab, respectively.

2.4 Western blot analysis of Japanese flounder IgM

Western blot was done following Lin et al. (2005). Briefly, samples were mixed with equal amounts of 2X SDS loading buffer and boiled for 10 min. After which, these (20 ul) were loaded in a 15% SDS-PAGE gel. A Precision Plus Protein Standard (Bio-Rad) was used as weight markers. Western blot transfer was then done in an ATTO clear blot

membrane at 110 mA for 1 hr. 1st antibody binding was done using a 1:5000 Pab-IgMs, while 2nd antibody used was an anti-mouse IgG-conjugated with alkaline phosphatase. Visualization of the reaction was carried out using a 5-bromo-4-chloro-3-indotyl phosphate/nitro blue tetrazolium alkaline phapatase (BCIP/NBT) substrate (Sigma-Aldrich, St/ Louis, MO, USA).

Sensitivity and tissue tropism analysis of the Pab-IgMs was carried out by western blot analysis of Japanese flounder tissues including heart, head kidney, tail kidney, liver, muscle, spleen and serum. Briefly, tissues were dissected out/extracted from 3 individual fish having about 10 cm total length. Solid tissues were lyzed by 1/3X PBS with sonication. Serum was separated from blood cells by centrifugation at 6,000 rpm for 15 min. Total protein concentration was measured as described above and standardized at 350 ug/ml (fish 1), 250 ug/ml (fish 2) and 150 ug/ml (fish 3). On the other hand, specificity of the Pab-IgMs was conducted by western staining whole tissue lysate of zebrafish, and serum of carp and tilapia at 5X dilution. Two fish samples per species were collected.

2.5 Immunohistochemistry analysis of Japanese flounder Pab-IgM

Immunohistochemical assay was done following Takano et al., 2007. Briefly, paraffin embedded or fresh smears of apparently healthy Japanese flounder kidney were formalin fixed in APS coated slides. These were then serially washed with xylene and decreasing concentration of Ethanol and then by 1X PBS. These were then incubated with hydrogen peroxide (H_2O_2) for 30 min. These were then incubated with IgM Pabs overnight inside a moist staining tray. After washing with 1X PBS, the 2nd antibody (Histofine: MAX-PO-MULTI) was introduced for 1 hr and then washed with 1 X PBS. The substrate, simple stain AEC, was used for visualization counterstained with hematoxylin. Resulting slides were mounted in glycerol and the cells were photographed using a microscope.

3. Results

3.1. IgM1 and IgM2 protein fragments

The recombinant IgM1 and IgM2 protein products, whether as inclusion bodies or in purified-renatured form, were ~ 19 kDa and ~ 10 kDa, respectively, which corresponds to their predicted molecular weights (Fig. 3) . Furthermore, both of the fragments were shown to be highly expressed by the bacterial expression system pET32-BL21 codon +.

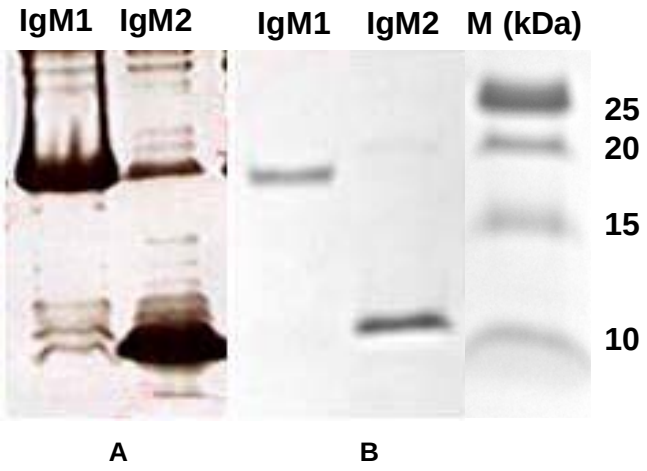

Figure 3. Recombinant Japanese flounder IgM fragment 1 and fragment 2. A) SDS-PAGE of IgM fragment 1 at ~19 kDa , and IgM fragment 2 at ~10 kDa. B) Western blots of purified IgMs using His-tag and dialysis at about the same molecular weight as the inclusion bodies.

3.2. Sensitivity and specificity of Pab-IgMs

Western blot analysis of tissues from 3 individual Japanese flounder samples at 3 different protein concentrations (350 ug/ml, 250 ug/ml and 150 ug/ml) shows that Pab - IgMs can detect the ~ 75 kDa IgM heavy chain in denatured form (Fig. 4). Moreover, Pab-IgM1 appeared to mark a secondary band that is ~ 70 kDa but not Pab-IgM2. Furthermore, tissue tropism analysis revealed that Japanese flounder IgM protein is equally expressed in heart, kidney and spleen but not in liver and muscle. In serum, IgM was detected to be in several different sizes.

Specificity analysis showed that the Pab-IgM1 is specific to Japanese flounder IgM with only apparent low cross-reaction in carp serum. In contrast, Pab-IgM2 showed strong cross-reactivity in the other 3 fish species analyzed (Fig. 5).

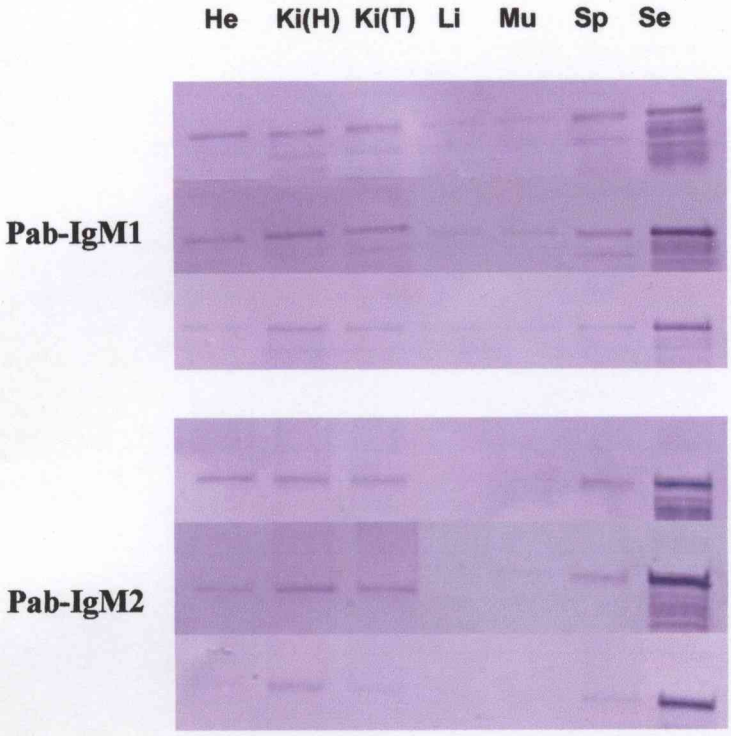

Figure 4. Western blot analysis of the constitutive expression of Japanese flounder IgM as detected by Pab-IgM1 and Pab-IgM2 in 3 fish samples under denatured conditions. He-heart, Ki (H)- Head kidney, Ki (T)- Tail kidney, Li- liver, Mu- muscle, Sp- spleen and Se-serum. Antibody is 1:5,000. 1- Fish 1, 350 ug/ml total protein; 2- Fish 2, 250 ug/ml total protein; 3- Fish 3, 150 ug/ml total protein.

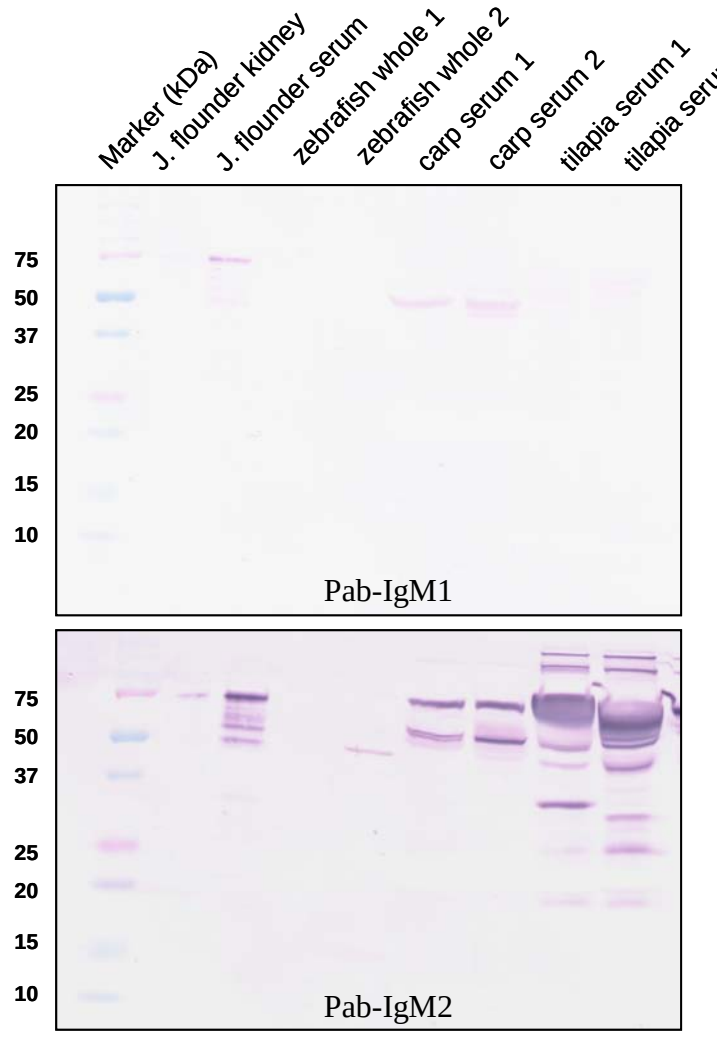

Figure 5. Specificity of Pab-IgM1 and IgM2 as shown by western blot analysis of Japanese flounder, zebrafish, carp and tilapia tissues/serum. Under denaturing condition at 15% SDS-PAGE gel. Pab dilution is 6 ul to 10 ml 1X blocking buffer.

3.3. Detection of IgM+ cells

Immunhistochemical staining revealed that Pab-IgM1 and IgM2 were able to detect IgM in normal kidney cells *in vivo* (Fig. 6.1 and 6.2). Thus, these can be considered as IgM+ cells. Moreover, it was shown that the Pab-IgM1 was clearly marking what appears to be a plasma cell (mature B cells) (Fig. 6.1B red arrow).

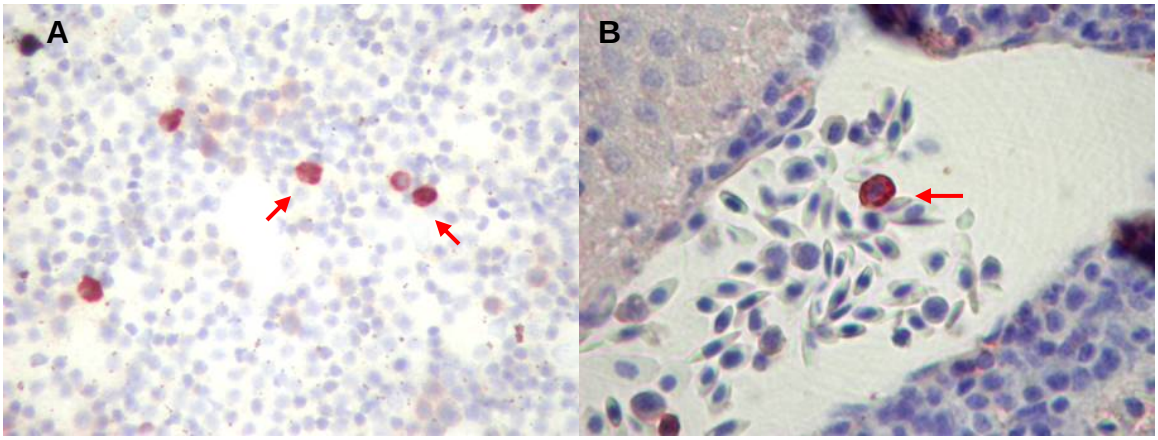

Figure 3.1. Immunohistochemical analysis of Japanese flounder kidney cells using IgM fragment 1 Pab. A) IgM positive kidney cells in kidney (red arrow) fresh mount. B) IgM positive cells in kidney (red arrow) paraffin mount, with labeled cell with morphology similar to plasma cells.

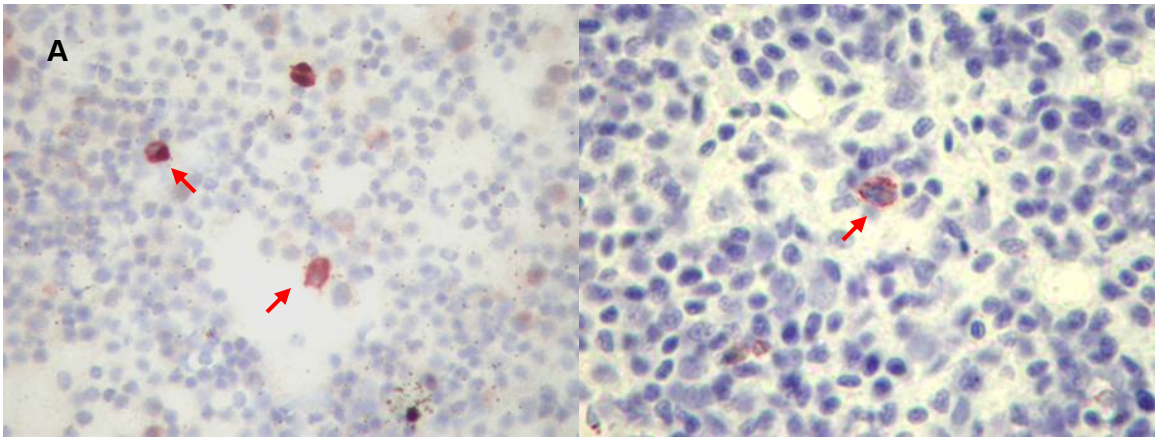

Figure 3.2. Immunohistochemical analysis of Japanese flounder kidney cells using IgM fragment 2 Pab. A) IgM positive kidney cells in kidney (red arrow) fresh mount. B) IgM positive cells in kidney (red arrow) paraffin mount.

4. Discussion

In this study, we produced a Japanese flounder IgM polyclonal antibodies (Pab-IgM1 and Pab-IgM2) derived from recombinant IgM proteins containing the fragments of the conserved region of the Heavy chain. The Japanese flounder IgM has been shown by an purified Ig-derived monoclonal antibody to be composed of 2 heavy (H) chains having

masses of about 77 kDa and 72 kDa, respectively (Jang et al., 2004). Our results revealed that both Pab-IgMs detected the same 77 kDa H chain and that Pab-IgM1 may well have been able to detect the 72 kDa H chain. The discrepancy in detecting ability between the Pab-IgMs is understandable as they were derived from 2 different protein fragments. Nevertheless, it shows that both Pabs are effective enough to bind to the selected IgM constant region.

We then checked for the concentration of IgM in various tissues in apparently healthy fish and perhaps as expected, IgMs were present in immune-related tissues such as the kidney and spleen, and to a lesser extent in muscle and liver. Interestingly, IgM concentration in heart was equal to that of kidney and spleen suggesting an important role of IgM in heart function. The serum IgM concentration is quite high (again as expected). The other lower bands detected in the serum may constitute different redox forms as has been suggested before (Kaatari et al., 1998) although this needs further investigation.

Pab-IgM1 but not Pab-IgM2 shows specificity in its detection capacity. Coupled with its ability to detect both H chains, it appears that Pab-IgM1 is more effective, thus more useful, than Pab-IgM2. This is further supported by the immunohistochemistry analysis where Pab-IgM1 was shown to strongly mark cells in kidney, in particular, morphologically-differentiated plasma cells. Likewise, immunostaining results suggest the ability of the Pab- IgMs to detect the presence of IgM+ cells in kidney, which have already been shown in the closely-related Atlantic halibut (Grove et al., 2006).

Taken together, our results suggest that the Pabs we developed, particularly Pab-IgM1, were comparable to published monoclonal antibodies for Japanese flounder (Jang et al., 2004) and Atlantic halibut (Grove et al., 2006). As such, they represent a cost-effective antibody resource that can be used in immunological studies and immunocompetence monitoring in Japanese flounder, a commercially important aquaculture species. Furthermore, the recombinant proteins produced may well be used for development of a more specific monoclonal antibody. Finally, we present a methodology that is very effective in producing sensitive and specific polyclonal antibodies.

Acknowledgement

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CHAPTER 6

General Conclusion

1. Immuno-hematopoietic cytokines and receptors in teleost fish

Three immuno-hematopoietic cytokine genes (CSF3, IL11b and MSH) were identified and their respective putative structures, orthology, paralogy and involvement in immune processes were established. These cytokines belonging to the IL6-cytokine subfamily, which possess the long chain 4 α-helical bundle structure, have been shown to be in duplicates (Type A and B) following the Whole Genome Duplication (WGD) theory and have correlated divergent expression and structures. In addition, the Type A members of these pleiotropic cytokines, thought to come from a single ancestral molecule (monophyletic), were shown to be the original genes and Type B as the duplicates.

The duplication of IL6-cytokines in teleost fish and their apparent divergent expression has various implications; 1) genes exhibiting high homology may not always translate to similarity in function, 2) teleost fish basic molecular processes appear to always constitute synergy of at least functional duplicate genes, 3) for application purposes such as development of vaccines/vaccine adjuvants in fish, combinatorial use of the duplicate genes may be more effective and 4) fish molecular processes such as immunity are far more complex compared to mammals than previously thought.

In this study, we also discovered a novel hematopoietic cytokine receptor named Japanese flounder gp 130 homologue (JfGPH) that was shown to be an ancestral molecule to some of the type 1 cytokine receptors and is involved in the Jak/STAT signal pathway.

Finally, a specific, cost-effective polyclonal Japanese flounder IgM antibody with comparable efficiency with monoclonal flounder IgM antibody has been produced that

can be useful for further immunological studies in a commercially important Japanese flounder.

2. Gene duplication in teleost fish

Present studies on orthologous teleost fish genes, albeit many are only up to expression studies, points to general similarity in function to higher vertebrates. Going forward, however, it is the fish-specific molecules and processes that would usher a new era in basic teleost fish immunological studies. The apparent duplication of many of the teleost fish genes adds to the complexity of fish immunology and could potentially reveal new mechanisms unheard of in the study of immunology. The availability of 5 fish genomic databases (for zebrafish, fugu, green spotted pufferfish, stickleback and medaka) will certainly fast track the study of these fish-specific phenomenon. This will allow for a better understanding of fish immunity as well as how this relate to the evolution of innate immunity in vertebrates.

3. Future studies

The detailed analysis of the innate immune-related molecules fish including their function and network will certainly generate new technologies that can be applied to improve aquaculture. For example, various pro-inflammatory cytokines can be used as potential for vaccine/adjuvant development. Previously, it was shown that Japanese flounder IL-1β can induce various cytokines, cell surface antigens, signal transduction genes, suggesting that IL-1β is a potential vaccine adjuvant (Emmadi et al., 2005).

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